



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Failures of nerve regeneration caused by aging or chronic denervation are rescued by restoring Schwann cell c-Jun

Citation for published version:

Wagstaff, LJ, Gomez-Sanchez, JA, Fazal, SV, Otto, GW, Kilpatrick, AM, Michael, K, Wong, LYN, Ma, KH, Turmaine, M, Svaren, J, Gordon, T, Arthur-Farraj, P, Velasco-Aviles, S, Cabedo, H, Benito, C, Mirsky, R & Jessen, KR 2021, 'Failures of nerve regeneration caused by aging or chronic denervation are rescued by restoring Schwann cell c-Jun', *eLIFE*, vol. 10. <https://doi.org/10.7554/eLife.62232>

Digital Object Identifier (DOI):

[10.7554/eLife.62232](https://doi.org/10.7554/eLife.62232)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

eLIFE

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Title: Failures of nerve regeneration caused by aging or chronic denervation are rescued by restoring Schwann cell c-Jun

Authors: Laura J. Wagstaff*, Jose A. Gomez-Sanchez*⁶ ¹Shaline V. Fazal, ²Georg W. Otto, ³Alastair M. Kilpatrick, Kirolos Michael, Liam Y.N. Wong, ⁴Ki H. Ma, Mark Turmaine, ⁴John Svaren, ⁵Tessa Gordon, ¹Peter Arthur-Farraj, ^{6,7}Sergio Velasco-Aviles, ^{6,7}Hugo Cabedo, Cristina Benito, Rhona Mirsky, Kristjan R Jessen

Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom

*These authors contributed equally to the work.

(1) John Van Geest Centre for Brain Repair, Department of Clinical Neurosciences, University of Cambridge, Cambridge, UK

(2) University College London Great Ormond Street Institute of Child Health, London WC1N1EH, UK

(3) School of Informatics, University of Edinburgh, Informatics Forum, Edinburgh EH8 9AB, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JR, UK

(4) Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin, USA

(5) Division of Plastic and Reconstructive Surgery, The Hospital for Sick Children, 555 University Ave, Toronto, Ontario, Canada, M5G1X8

(6) Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC, 03550 San Juan de Alicante, Spain

(7) Hospital General Universitario de Alicante, ISABIAL, Pintor Baeza 11, 0310 Alicante, Spain

Submitting author: Kristjan R. Jessen Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT.

k.jessen@ucl.ac.uk

Corresponding author: Kristjan R. Jessen Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E6BT.

k.jessen@ucl.ac.uk

ABSTRACT

After nerve injury, myelin and Remak Schwann cells reprogram to repair cells specialized for regeneration. Normally providing strong regenerative support, these cells fail in aging animals, and during chronic denervation that results from slow axon growth. This impairs axonal regeneration and causes significant clinical problems. In mice, we find that repair cells express reduced c-Jun protein as regenerative support provided by these cells declines during aging and chronic denervation. In both cases, genetically restoring Schwann cell c-Jun levels restores regeneration to control levels. We identify potential gene candidates mediating this effect and implicate Shh in the control of Schwann cell c-Jun levels. This establishes that a common mechanism, reduced c-Jun in Schwann cells, regulates success and failure of nerve repair both during aging and chronic denervation. This provides a molecular framework for addressing important clinical problems, suggesting molecular pathways that can be targeted to promote repair in the PNS.

59 Among mammalian systems, peripheral nerve is often hailed as a prime example of a tissue
60 with a striking regenerative potential. Nerve injury triggers the reprogramming of myelin and
61 non-myelin (Remak) Schwann cells to adopt a repair Schwann cell phenotype specialized to
62 support regeneration, and injured neurons activate a gene program that facilitates axon
63 growth. Yet, paradoxically, the clinical outcome of nerve injuries remains poor, and nerve
64 damage constitutes a significant clinical and economic burden. Remarkably, treatment of
65 nerve injuries has not advanced significantly for decades (Furey et al. 2007; Jonsson et al.
66 2013; reviewed in Fu and Gordon 1995; Boyd and Gordon 2003a; Hoke 2006; Allodi et al.
67 2012; Scheib and Hoke 2013; Doron-Mandel et al. 2015; Jessen and Mirsky 2016; Fawcett
68 and Verhaagen 2018; Jessen and Arthur-Farraj 2019).

69 The question of why a potentially regenerative tissue fails to respond effectively to injury and
70 ensure clinical recovery is important both for promoting nerve repair, and also more
71 generally. A number of other systems with experimentally established regenerative capacity,
72 e.g. skin, heart and pancreatic islets, also fail to show clinically useful regenerative response
73 to tissue damage (Cohen and Melton, 2011; Eguizabal et al. 2013; Jessen et al. 2015).

74 In the case of peripheral nerves, recent work has highlighted two important factors that
75 prevent full expression of their regenerative potential. One is the age of the animal at the
76 time of injury, increasing age resulting in a marked decrease in regeneration. The other is
77 the adverse effect of chronic denervation on the nerve distal to injury, since this tissue
78 gradually loses the capacity to support axon growth as it lies denervated during the often
79 extensive time it takes regenerating axons to reach their targets. These two problems turn
80 out to involve a common factor, namely a repair Schwann cell failure, since both during
81 aging and chronic denervation, the denervated Schwann cells in the distal stump undergo
82 molecular and morphological changes that result in a striking functional deterioration of
83 these important drivers of axonal regeneration (reviewed in Verdu et al 2000; Sulaiman and
84 Gordon 2009; Painter 2017; Jessen and Mirsky 2019).

In the present work, we have tested whether the dysfunction of repair Schwann cells in these two apparently unrelated situations relates to a common factor, namely a failure to activate or maintain high levels of the transcription factor c-Jun. That this might be so, is based on our previous finding that c-Jun, which is upregulated in Schwann cells in injured nerves, is a global amplifier of the repair Schwann cell phenotype (Arthur-Farraj et al. 2012; reviewed in Jessen and Mirsky 2016; 2019; Jessen and Arthur-Farraj 2019), and on subsequent findings showing that enhanced Schwann cell c-Jun promotes regeneration, both through nerve grafts and *in vitro* (Arthur-Farraj et al. 2012; Huang et al. 2015; 2019).

The age-dependent decline in regenerative capacity of human and animal nerves is well established (Pestronk et al. 1980; Tanaka and Webster 1991; Tanaka et al. 1992; Graciarena et al. 2014; reviewed in Vaughan 1992; Verdú et al. 2000; Ruijs et al. 2005). This is associated with a reduced initial inflammatory response followed by enhanced chronic inflammation (Scheib and Höke 2016; Büttner et al. 2018). Interestingly, diminished regeneration is not caused by age-dependent changes in neurons. Rather, aging results in subdued activation of the repair Schwann cell phenotype after injury, including reduced c-Jun expression, resulting in regeneration failure (Painter et al. 2014; reviewed in Painter 2017).

The other major barrier to repair that we consider here is caused by long-term denervation of nerves distal to injury. This is an important issue in human nerve regeneration (Ruijs et al. 2005), and has been studied in some detail in rats, revealing that chronic denervation results in reduced expression of repair-associated genes including *Gdnf*, *Bdnf*, *Ntf3* and *Ngfr*, accompanied by a dramatic reduction in the ability of denervated distal stumps to support regeneration even of freshly transected axons (Fu and Gordon 1995; You et al. 1997; Sulaiman and Gordon 2000; Höke et al. 2002; Michalski et al. 2008; Eggers et al. 2010). There is direct evidence for a comparable deterioration of repair cells and repair capacity during chronic denervation of human nerves (Wilcox et al. 2020; reviewed in Ruijs et al. 2005). Chronic denervation also results in reduced repair cell numbers and shortening of

repair cells (Benito et al. 2017; Gomez-Sanchez et al. 2017; reviewed in Jessen and Mirsky 2019). Thus, the repair phenotype is not stable, but fades with time after injury, thereby contributing to the poor outcome after nerve damage in humans.

Schwann cell reprogramming after nerve injury involves up-regulation of trophic factors and cytokines, activation of EMT genes, and myelin autophagy for myelin clearance and down-regulation of myelin genes (Brushart et al. 2013; Arthur-Farraj et al. 2017; Clements et al. 2017; reviewed in Gröthe et al. 2006; Chen et al 2007; Gambarotta et al. 2013; Glenn and Talbot 2013; Jessen and Mirsky 2016; Boerboom et al. 2017; Jessen and Arthur-Farraj 2019; Nocera and Jacob 2020). Myelin and Remak Schwann cells also increase in length by two-to-three fold and often branch as they convert to repair cells and form regeneration tracks, Bungner bands, that guide regenerating axons (Gomez-Sanchez et al. 2017). The molecular signals involved in the decline of these repair-supportive features during aging and chronic denervation have not been known.

The transcription factor c-Jun regulates the reprogramming of myelin and Remak cells to repair cells by accelerating the extinction of myelin genes, promoting myelin breakdown, and by amplifying the up-regulation of a broad spectrum of repair-supportive features, including the expression of trophic factors. Accordingly, genetic removal of c-Jun from Schwann cells results in functionally impaired repair cells and regeneration failure (Arthur-Farraj et al. 2012; Fontana et al., 2012; reviewed in Jessen and Arthur-Farraj 2019).

Here we provide evidence that a common molecular mechanism, the dysregulation of c-Jun in Schwann cells, is central to two major categories of regeneration failure in the PNS. The high levels of Schwann cell c-Jun triggered by nerve injury in young animals are not achieved in older ones, and, irrespective of age, the elevated c-Jun expression seen after injury steadily decreases during long-term denervation. Importantly we show that in both models of regeneration failure, genetically restoring Schwann cell c-Jun levels in vivo also restores regeneration rates to that in controls. By establishing c-Jun as an important regulator of the success and failure of nerve repair during aging and chronic denervation this

observation provides a common molecular framework for addressing an important clinical problem, and suggests molecular pathways that can be targeted to promote repair in the PNS

RESULTS

IN AGING ANIMALS, MAINTAINING C-JUN LEVELS IN SCHWANN CELLS REVERSES AGE-RELATED DEFECTS IN NERVE REGENERATION

Age-dependent failure of nerve regeneration is accompanied by subdued elevation of c-Jun, a major regulator of the repair cell phenotype (Painter et al. 2014). To test whether this reduction in c-Jun controls the reduced capacity of these cells to support axon growth, we first compared c-Jun upregulation in young and older WT mice (Fig.1 A). Four days after transection, c-Jun protein levels in the distal nerve stump in aged mice (8-10 month) were found to be ~ 50% lower than in young (6-8 week) mice.

To determine the functional significance of this, we studied *Mpz^{Cre+};R26c-Junstop^{fl/+}* mice (referred to as c-Jun OE/+ mice), which we generated previously (Fazal et al. 2017). In these mice, c-Jun levels are enhanced in Schwann cells only. In Western blots of uninjured adult sciatic nerves of c-Jun OE/+ mice, c-Jun is elevated about seven fold compared to WT. While there is a modest reduction in myelin thickness, nerve architecture and Schwann cell morphology are normal (Fazal et al. 2017). We found that in c-Jun OE/+ mice, the age-dependent decline in c-Jun protein levels after sciatic nerve cut was prevented, and that c-Jun levels in the distal stump of young WT and aging c-Jun OE/+ mice were similar by Western blots 4 days after cut (Fig.1 A). At the mRNA level, a non-significant trend towards lower c-Jun expression was seen in 3 day cut nerves of aged WT nerves, while there was a significant elevation of c-Jun mRNA in cut c-Jun OE/+ nerves as expected (Fig.1 B).

c-Jun levels seen in Western blots were confirmed in immunofluorescence experiments on 3 day cut nerves, using Sox10 antibodies to selectively identify Schwann cell nuclei, and c-Jun

antibodies (Fig.1C). In WT mice, older nerves contained fewer c-Jun positive Schwann cell nuclei and the labeling of the c-Jun positive nuclei was weaker, compared to young nerves. In aged c-Jun OE/+ nerves nuclear c-Jun was restored to levels similar to those in young WT nerves.

Regeneration in young and aged WT mice and aged c-Jun OE/+ mice was compared using neuron back-filling, a method that provides an optimal measure of regenerative capacity by determining the number of neuronal cell bodies have regenerated axons through a nerve at a measured distance distal to injury (Fig. 1 Figure Supplement 1) (Novikova et al.1997; Boyd and Gordon 2003b; Catapano et al. 2016). Four days after sciatic nerve crush, retrograde tracer was applied to the distal stump 7 mm from the crush site. Seven days later, the animals were sacrificed and the number of back-filled DRG and spinal cord motor neurons were counted. The results were comparable for both neuronal populations (Fig.1 D, E). The number of neurons regenerating through the distal stump of aged WT mice was reduced by about 50% compared to young mice. In aged c-Jun OE/+ mice on the other hand, regeneration of DRG and motor neurons was restored to levels similar to those in young WT nerves.

In confirmation, counts of CGRP+ fibers in the sciatic nerve were performed 3 days after nerve crush, 3mm from the injury site (Fig.1 F). The number of fibers were reduced in aged WT nerves compared to young ones, but increased in the aged c-Jun OE/+ nerves.

These experiments confirm that the failure of repair cell function in older animals is accompanied by failure to fully upregulate c-Jun in Schwann cells after injury (Painter et al. 2014). Importantly, restoration of c-Jun elevation selectively in Schwann cells to that seen in young animals is sufficient to restore nerve regeneration to youthful levels.

A MOUSE MODEL OF DISTAL STUMP DETERIORATION

We established a model of chronic denervation in mice, since previous studies have been carried out in rats. Sciatic nerves were cut followed by deflection of the proximal stump to leave the distal stump un-innervated for 1 week (short-term denervation) or 10 weeks (chronic denervation). mRNA levels for genes associated with denervated Schwann cells (*S100b*, *Ngfr*, *Gdnf*, and *sonic hedgehog (Shh)*) declined substantially between 1 and 10 weeks of denervation (Fig.2 A). Western blots of p75NTR (Ngfr) in the distal stump showed a rise to a maximum 1 week after injury and a decline thereafter to < 50% of peak levels at 10 weeks (Fig. 2 B) in line with that seen in rat (You et al. 1997).

Regeneration through freshly cut and long-term denervated nerve stumps were compared by back-filling of spinal cord motor neurons. For this, acutely cut (immediate repair) or 10 week denervated tibial distal stumps were sutured to freshly cut peroneal nerves (Fig.2 Figure Supplement 1 Two weeks later, Fluorogold retrograde tracer was applied to the tibial stumps 4mm distal to the suturing site. One week after the application of tracer, the number of retrogradely labeled motor neurons in the spinal cord was counted. Only about half as many spinal cord motor neurons projected into the 10 week denervated stumps compared to the acutely transected stumps (Fig.2 C).

Failure of regeneration through 10 week denervated stumps was confirmed by immunohistochemistry and counting of neurofilament positive axons in similar experiments. Ten week denervated stumps contained many fewer regenerating neurofilament labeled axons than nerve stumps sutured immediately after transection (Fig.2 D).

Counting back-filled neurons following immediate repair or repair 1 week after transection, revealed a similar number of regenerating neurons (Fig.2 E). This shows that the capacity of the distal stump to support regeneration declines between 1 and 10 weeks of denervation.

These experiments established a baseline for studying the effects of prolonged denervation on the capacity of mouse repair Schwann cells to support neuronal regeneration.

c-JUN IS DOWN-REGULATED IN CHRONICALLY DENERVATED SCHWANN CELLS

We determined whether decline in Schwann cell c-Jun expression was involved in regeneration failure caused by chronic denervation, as seen during aging. Measuring c-Jun protein levels in distal stumps showed strong elevation 3 days and 1 week after sciatic nerve cut followed by a decline to ~40% of 1 week levels at 10 weeks (Fig.3 A). We verified that the c-Jun shown in these Western blots represented c-Jun in Schwann cells, using mice with conditional c-Jun inactivation selectively in Schwann cells (Arthur-Farraj et al. 2012) (Fig.3 B). Further, 1 and 10 week denervated stumps were compared using double immunofluorescent labeling with c-Jun antibodies and Sox10 antibodies to selectively identify Schwann cell nuclei. Ten week denervated stumps showed many more c-Jun negative, Sox10 positive nuclei, and the c-Jun labeling, where present, was also weaker (Fig.3 C).

The decline in c-Jun and p75NTR expression after long-term denervation *in vivo* was mimicked in purified Schwann cells *in vitro*. By Western blots, cells that had been maintained ~6 weeks *in vitro* (9 passages), contained less c-Jun and p75NTR protein compared to cells maintained ~10 days *in vitro* (2 passages) (Fig.3 D). The levels of c-Jun mRNA also declined in long-term cultures (Fig.3 E) The reduction in c-Jun in Schwann cell nuclei was confirmed using double immunofluorescent labeling with c-Jun and Sox 10 antibodies to identify Schwann cells (Fig.3 F). These *in vitro* experiments suggests that the decline in c-Jun and p75NTR during chronic denervation is not driven by endoneurial signals.

C-JUN DOWN-REGULATION DURING CHRONIC DENERVATION IS PREVENTED IN C-JUN OE/+ MICE

The decline in c-Jun levels at the same time as repair cells lose capacity to support regeneration raises the questions of whether the functional deterioration of these cells is

partly a consequence of c-Jun reduction, and whether repair cells, and regeneration through chronically denervated nerves, would be maintained if c-Jun reduction was prevented.

We addressed this using the c-Jun OE/+ mice examined earlier in experiments on aging (previous section; Fazal et al. 2017). The mice were 6-8 weeks old at the time of injury, corresponding to young mice in the study on aging. One and 3 week denervated distal stumps of c-Jun OE/+ and WT mice contained similar c-Jun protein levels. However, at 10 weeks, when c-Jun had declined in WT mice, c-Jun was maintained in c-Jun OE/+ mice at levels similar to those at 1 week (Fig.4 A). This was confirmed by double label c-Jun/Sox10 immunofluorescence (Fig.4 B). This showed similar c-Jun nuclear labeling in 1 week denervated stumps of WT and c-Jun OE/+ mice, while at 10 weeks, WT nerves showed a reduced number of c-Jun positive nuclei and decreased labeling intensity. This decrease was prevented in c-Jun OE/+ nerves.

These experiments indicate that in 1-3 week cut nerves, the maximum capacity of Schwann cells to express c-Jun protein is already reached in the WT, both genotypes showing a similar 80-100 fold elevation after injury. During chronic denervation, these high expression levels fall substantially in WT nerves, but not in c-Jun OE/+ nerves. The c-Jun OE/+ mice can therefore be used to test whether the regeneration failure induced by chronic denervation is due to the failure to maintain high c-Jun protein levels.

MAINTAINING C-JUN LEVELS DURING CHRONIC DENERVATION PREVENTS REGENERATION FAILURE

Regeneration thorough chronically denervated distal stumps of WT and c-Jun OE/+ mice was compared using neuron back-filling. Acutely cut common peroneal nerves were sutured to acutely cut or 10 week denervated tibial nerves and allowed to regenerate for 2 weeks prior to application of Fluorogold retrograde tracer. In WT mice, the number of DRG neurons projecting through 10 week cut nerves was only about half that projecting through acutely cut

267 nerves. In 10 week cut c-Jun OE/+ nerves, however, this drop was not seen (Fig.5 A).
268 Similar results were obtained for spinal cord motor neurons (Fig.5 B). Thus, both DRG and
269 motor neurons regenerated as well through 10 week denervated c-Jun OE/+ nerves as they
270 did through acutely cut WT nerves, suggesting that c-Jun OE/+ Schwann cells maintain their
271 capacity to support regenerating neurons despite chronic denervation. In confirmation,
272 regeneration into 10 week denervated distal stumps of the tibial nerve was examined with
273 neurofilament staining, 1 week after the stumps were sutured to freshly cut peroneal nerves.
274 Nearly 3 times more fibers were found in 10 week denervated c-Jun OE/+ nerves compared
275 to 10 week denervated WT nerves (Fig.5 C). The number of fibers found in WT nerves after
276 immediate repair is shown in Fig. 2 D.

277 We verified that the back-filling paradigm worked as expected in c-Jun OE/+ mice. First,
278 peroneal nerves in WT and c-Jun OE/+ mice were transected followed by immediate
279 application of tracer to the injured proximal stump. The number of back-filled DRG and motor
280 neurons in c-Jun OE/+ mice was similar to that in WT animals (Fig.5 D, E). This does not
281 measure regeneration, but indicates that a comparable number of DRG and motor neurons
282 project through the normal uninjured peroneal nerve in the two mouse lines. Second,
283 although c-Jun levels in WT and c-Jun OE/+ mice diverge after 10 weeks of denervation,
284 they are high, and similar, early after injury, when the capacity of WT nerves to support
285 regeneration is optimal. In line with this, the regeneration support provided by WT and c-Jun
286 OE/+ nerves was similar early after injury. Thus, in back-filling experiments no significant
287 difference was seen between WT and c-Jun OE/+ mice in the numbers of DRG or motor
288 neurons that regenerated through acutely transected distal stumps (immediate repair) when
289 the tracer was applied 2 weeks after transection/repair. (Fig.5 F and not shown). Similarly,
290 comparable numbers of back-filled motor neurons were obtained in WT and c-Jun OE/+
291 mice when back-filling was used to quantify regeneration 5 days after sciatic nerve crush
292 (Fig.5 G).

Together, these experiments indicate that under the conditions prevailing in the distal stump of cut nerves, Schwann cells are unable to maintain high c-Jun levels in the long term. The resulting gradual reduction in c-Jun leads to deterioration of repair cell function, causing regeneration failure, a failure that can be corrected by restoring c-Jun levels.

IMPROVED REGENERATION IN C-JUN OE+/- MICE IS UNLIKELY TO RELATE TO ALTERED CELL NUMBERS

To test whether the differential regeneration rates could be due to altered cell numbers, Schwann cell, macrophage and fibroblast nuclei were counted in tibial nerves by electron microscopy.

In experiments comparing young and aging animals, cells in the distal stump were counted 4 days after nerve cut without regeneration. Schwann cell and macrophage numbers and density were similar irrespective of genotype or age (Fig.6 A-C). There was some decrease in fibroblast density in aged mice, but density and numbers were similar irrespective of genotype (Fig.6 D, E). In aged mice, transverse profiles of the tibial nerve were larger mainly because of increased endoneurial connective tissue, but no difference was seen between the genotypes (Fig.6 F).

In the experiments on chronic denervation, cells were counted in the distal stumps 2 and 10 weeks after cut without regeneration 5 mm from the injury site. There were ~250 Schwann cell nuclei in 2 week stumps, representing a ~2.5 fold increase from uninjured nerves (Fazal et al. 2017) (Fig.6 G). In the WT, Schwann cell numbers declined by ~30% during chronic denervation. This drop was prevented in c-Jun OE/+ nerves (Fig.6 G). The density of macrophages was similar irrespective of genotype or length of denervation (Fig.6 H), with numbers declining, irrespective of genotype, during chronic denervation (Fig.6 I). Two weeks after injury, fibroblast density in WT and c-Jun OE/+ mice was similar. Fibroblast density was elevated in 10 week stumps of WT, but not c-Jun OE/+, mice (Fig.6 J). Fibroblast numbers

remained unchanged between genotypes following denervation (Fig.6 K). Chronically denervated stumps of both genotypes had reduced nerve area, although this difference was less evident in c-Jun OE/+ nerves (Fig.6 L).

These counts indicate that altered cell numbers are not a significant reason for improved regeneration in aged c-Jun OE/+ nerves. In chronically denervated nerves, the normal loss of Schwann cells in WT nerves is prevented in c-Jun OE/+ nerves. Even in 10 week WT stumps, however, cell numbers remain well above those in uninjured nerves (see Discussion).

SHH SIGNALING SUPPORTS C-JUN EXPRESSION.

Seeking mechanisms that promote c-Jun expression in denervated Schwann cells, we considered signaling by Shh, a gene that is not expressed in developing Schwann cells or in intact nerves, but strongly up-regulated in repair Schwann cells after injury (Lu et al. 2000; Zhou et al. 2000; Arthur-Farraj et al. 2012; Hsin-Pin et al. 2015). First, we analysed Shh cKO mice, in which Shh is electively inactivated in Schwann cells and in which nerve ultrastructure appears normal as expected (J Svaren unpublished). We found that in the mutants, c-Jun protein and phosphorylated c-Jun were decreased in the distal stump of 7 day transected sciatic nerve, (Fig.7 A, B). Levels of p75NTR protein, which is positively regulated by c-Jun in Schwann cells (Arthur-Farraj et al. 2012), were also reduced 7 days after injury in the mutants (Fig.7 C). Substantiating these observations, two Shh signaling agonists, SAG and purmorphamine, upregulated c-Jun protein in purified Schwann cell cultures (Fig.7 D, E). This was confirmed using c-Jun/Sox10 double immunolabeling (Fig.7 F). SAG also increased the expression of two c-Jun target genes that promote nerve regeneration *Bdnf* and *Gdnf* (Fig.8 A, B). Further, SAG promoted another effect of c-Jun, that of enhancing the elongated bi-or tri-polar shape in vitro that reflects the elongation and

branching of repair cells in vivo (Arthur-Farraj et al. 2012; Gomez-Sanchez et al. 2017) (Fig.8 C).

After injury, Shh-dependent enhancement of c-Jun is likely to be mediated by Shh derived from Schwann cells, which are the major source of Shh in injured nerves. In support of such an autocrine hedgehog signaling loop, cyclopamine alone, which blocks cellular responses to Shh, down-regulated c-Jun protein and sharply suppressed c-Jun phosphorylation in cultured Schwann cells (Fig.8 D).

Together these observations show that Shh promotes Schwann cell c-Jun expression in vitro and in vivo, and support the idea that injury triggers an autocrine Shh signaling loop to elevate c-Jun in repair cells.

ANALYSIS OF GENE EXPRESSION IN DISTAL NERVE STUMPS OF WT AND C-JUN OE/+ MICE

Young and aging mice. RNA sequence analysis was performed on uninjured and 3 day cut sciatic nerves of young (6-8 weeks) and aged (11-12 months) WT mice, and aged c-Jun OE/+ mice. Global gene expression was analysed comparing (i) uninjured nerves, (ii) 3d cut nerves, and (iii) the injury response (3d cut vs uninjured) in young and aged mice (Fig.9 - Figure Supplement 1 A).

In uninjured WT nerves, out of 15,995 genes present, 1,477 genes were differentially expressed between young and aged mice. Of these, 1,154 were up-regulated and 323 down-regulated in aging mice compared to young ones (FC >2; and FDR 0.05) (Supplementary File 1 A; Supplementary File 2 A). We tested whether the 173 genes we previously identified as c-Jun regulated injury genes (Arthur-Farraj et al. 2012) were implicated in these age-dependent differences. In the present data set, 138 of the 173 genes were present (Supplementary File 3). They showed a strong enrichment among the 1477 genes differentially expressed between young and aging mice (Fig.9 A).

In 3d cut WT nerves, of 17,334 genes present, 398 genes were differentially expressed between young and aging mice. Of these, 268 were up-regulated and 130 down-regulated in aging nerves (Supplementary File 1 B; Supplementary File 2 B). This gene set contains candidate genes responsible for the difference in regeneration support provided by young and aged Schwann cells (Painter et al. 2014 and present experiments). In agreement with Painter et al. (2014), trophic factors such as GDNF, BDNF, NGF, erythropoietin and FGF were not among the differentially expressed genes. This suggests that expression of trophic factors often implicated in regeneration may not explain different regeneration between young and aged mice. The 138 c-Jun regulated injury genes (Supplementary File 3; Arthur-Farraj et al. 2012) were highly enriched among the age-regulated genes (Fig.9 A).

Examining the injury response (3 d cut vs uninjured nerve), 822 genes showed significant difference when young and aging WT mice were compared (Supplementary File 1 C; Supplementary File 2 C). The 138 c-Jun regulated injury genes were strongly enriched among these 822 genes (Fig.9 A).

Gene set enrichment analysis (GSEA) on the above conditions showed that the 138 c-Jun genes were highly enriched among the genes down-regulated in aging uninjured nerves and in aging 3 day cut nerves (Fig. 9 - Figure Supplement 1 B, C). When the injury response (3d cut vs uninjured) of young and aging WT mice was analysed, the strongest enrichment of c-Jun genes was observed in genes upregulated in young nerves (Fig. 9 - Figure Supplement 1 D). While c-Jun genes are also upregulated in aged nerves post-injury, their enrichment was not as high as in young nerves.

These correlations between enrichment of c-Jun regulated genes and Schwann cell age suggest that the c-Jun regulated repair program is disproportionately vulnerable during the aging process.

Gene ontology (GO) analysis showed that in aged uninjured WT nerves, down-regulated genes were largely involved in lipid metabolism, as well as myelination, while genes involved

in the immune system were prominent among those up-regulated (Fig.9 B, C; reviewed in Melcangi et al. 1998; 2000; Büttner et al. 2018). Similar analysis of the injury response (3 day cut vs uninjured) showed reduced activation of immune genes in aged WT nerves (Scheib and Höke 2016; Büttner et al. 2018). In aged nerves, MAPK pathways were also suppressed while lipid metabolism and Schwann cell differentiation genes were enhanced (Fig.9 D, E). Together this indicates suppressed Schwann cell reprogramming and repair cell activation in nerves of older WT mice. Testing the effects of enhanced c-Jun expression on the aged injury response, we found that pathways associated with protein processing and maturation were up-regulated in aged c-Jun OE/+ nerves compared with aged WT nerves (Fig.9 F).

To further determine genes that may contribute to the restoration of regeneration in aged c-Jun OE/+ mice, the injury responses in young and aged WT mice and aged c-Jun OE/+ mice were compared (Fig.9 G). Of particular interest are the 303 genes that show significant injury response in young WT mice but not in aging WT mice, but are again significantly regulated by restoring c-Jun to youthful levels in aging c-Jun OE/+ mice (Supplementary File 2 D).

Among the 138 c-Jun regulated genes, we looked for a correlation between a failure and restoration of gene expression on the one hand, and failure and restoration of regeneration on the other. In 3 day cut aged WT nerves, where regeneration fails, 16 c-Jun regulated genes were differentially expressed compared to 3 day cut young WT nerves. Four of these, Aqp5, Gpr37L1, Igfbp2, and Olig1, were restored in aged c-Jun OE/+ nerves, where regeneration is restored (Fig 9 H). Thus, in aging mice, both regeneration failure and the expression defect of these four genes was restored to levels in young mice, by elevating c-Jun levels.

Chronic denervation. Gene expression was examined in uninjured nerves and in 1 and 10 week cut sciatic nerves of WT and c-Jun OE/+ mice (Fig. 10 - Figure Supplement 1 A). Expression of 1581 genes changed significantly during chronic denervation (Supplementary File 4; Supplementary File 2 E). In 10 week cut nerves, 601 of these genes were down-

regulated, including genes associated with repair cells such as *Gdnf*, *Shh* and *Ngfr*, while 980 genes were up-regulated. The 138 c-Jun regulated genes showed a highly significant 5.8 fold enrichment ($p=2.2 \times 10^{-16}$) among the 1581 genes regulated during chronic denervation. GSEA enrichment analysis showed that c-Jun genes were some of the most down-regulated genes during chronic denervation. (Fig.10 - Figure Supplement 1 B).

GO analysis showed that the major genes down-regulated during chronic denervation in WT nerves involved the cell cycle, DNA replication and repair. Glial cell differentiation genes and MAPK pathways, potential activators of c-Jun, were also suppressed (Fig.10 A). Chronic denervation involved a prominent up-regulation of neuro-glia signaling genes (chiefly related to GABA and adrenergic signalling), but also negative regulators of differentiation, Notch and cAMP signaling (Fig.10 B). To test the effects of maintaining c-Jun protein levels during the 10 week chronic denervation, we identified genes differentially expressed between 10 week cut WT and c-Jun OE/+ nerves (Fig.10 C). This showed strong up-regulation, in c-Jun OE/+ mice, of pathways involved in PNS and Schwann cell development and differentiation.

To further determine genes that may contribute to the restoration of regeneration in aged c-Jun OE/+ mice, the injury response in the three situations analysed in the regeneration experiments, WT 1 week, WT 10 weeks and c-Jun OE/+ 10 weeks, was compared (Fig.10 D). A point of interest are the 227 genes that showed an injury response in WT 1 week nerves and in c-Jun OE/+ 10 week nerves, both of which show fast regeneration, but no injury response in WT 10 week nerves, where regeneration is slow (Supplementary File 2 F).

As when studying aged mice, we looked among the 138 c-Jun regulated injury genes for candidates involved in decreased regeneration in 10 week cut WT nerves and the restoration of regeneration in 10 week cut c-Jun OE/+ nerves. Fifty of the 138 genes changed expression during chronic denervation in WT nerves, where regeneration is poor. In chronically denervated c-Jun OE/+ nerves, where regeneration is restored, expression levels were restored, completely or partially, in the case of five of these genes, *Cxcl5*, *Egfl8*, *Gas2l3*, *Megf10* and *Pcdh20* (Fig.10 E). These correlations provide a basis for considering

these genes as candidates down-stream of c-Jun for involvement in the restoration of regeneration in chronically denervated nerves of c-Jun OE/+

DISCUSSION

The present results indicate that reduced expression of c-Jun is an important factor in the repair cell failures seen during aging and chronic denervation. In both situations, Schwann cells of injured nerves fail to achieve or maintain high c-Jun levels, and in both cases, correction of c-Jun expression restores regeneration deficits. This highlights the importance of c-Jun in the function of repair Schwann cells, provides a common molecular link between two apparently unrelated problems in nerve repair, and points to manipulation of c-Jun regulated pathways as a potential route for improving the outcome of nerve injuries.

By using neuron back-filling this study provides a direct quantitative measure of neuronal regeneration capacity in vivo and how this is controlled by Schwann cell c-Jun levels. It also opens new questions that remain to be investigated. In particular, to what extent does c-Jun in Schwann cells determine other factors that are also important for repair. This includes the length of time neurons are able to sustain axon growth after injury, sprouting, axonal misrouting, targeting and synapse re-formation.

A previously identified gene set regulated by c-Jun in injured nerves (Arthur-Farraj et al. 2012), was found to be highly enriched among the genes affected by aging or chronic denervation in WT mice. The expression of a small group of genes was also positively correlated both with c-Jun levels and regeneration, suggesting that their role in Schwann cells or other cells in the nerve merits further study. In aging mice this encompasses Aqp5, Gpr37L1, Igfbp2 and Olig1, all of which have been studied in glial cells. Igfbp2 promotes phosphorylation of Akt, a pathway that is linked to Schwann cell proliferation and differentiation (reviewed in Ma et al. 2015; Boerboom et al. 2017; Jessen and Arthur-Farraj 2019). Gpr37L1 is a receptor for prosaposin and prosapeptide (Meyer et al. 2013). In

Schwann cells prosaposin phosphorylates MAPK (Hiraiwa et al. 1997) and prosaposin is secreted after nerve injury, facilitating regeneration (Hiraiwa et al. 1999). In experiments on chronic denervation this gene group encompasses Cxcl5, Egfl8, Gas2l3, Megf10 and Pcdh20. All of these genes were previously shown to be up-regulated in Schwann cells after injury (Zhang et al. 2011; Tanaka et al. 2013; Weiss et al. 2016; reviewed in Ma et al. 2016; Brosius Lutz et al. 2017). Cxcl5 activates STAT3 (Zhang et al. 2011), a transcription factor that we have shown to be important for maintaining repair cells during chronic denervation (Benito et al. 2017). Gas2l3 has a role in the cell cycle, and Megf10 in phagocytosis (Wolter et al. 2012; Chung et al. 2013).

Since c-Jun levels in injured nerves are a major determinant of effective repair, it is important to identify signals that control c-Jun expression. The present results suggest that Shh has a role in this process. In injured nerves of Shh cKO mice, there is reduced c-Jun activation and diminished Schwann cell expression of the c-Jun target p75NTR. In purified Schwann cells, application of Shh elevates c-Jun, while cyclopamine alone suppresses c-Jun. Shh also promotes Schwann cell elongation. Further, during chronic denervation, Shh expression, like that of c-Jun, is substantially reduced. Previous work also implicates Shh signaling in repair. Shh is up-regulated in Schwann cells after injury (Hashimoto et al. 2008; Arthur-Farraj et al. 2012; Yamada et al. 2018), and exposure to Shh improves nerve regeneration in various settings (Pepinsky et al. 2002; Bond et al. 2013; Martinez et al. 2015; Yamada et al. 2018). Inhibition of Shh signaling reduces Schwann cell expression of BDNF, motor neuron survival after injury and axon regeneration (Hashimoto et al. 2008; Yamada et al. 2020), and a molecular link between Shh signaling and Jun activation has been established in various cell lines (Laner-Plamberger et al. 2009; Kudo et al. 2012). Further in vivo experiments using Shh cKO mice as well as Shh agonists and antagonists are needed to conclusively determine the involvement of Shh in regeneration. At present, however, the data presented here and previous work are consistent with the existence of an autocrine Shh signaling loop activated by injury to promote expression of c-Jun and the repair cell phenotype.

In c-Jun OE/+ mice, we considered whether restoration of c-Jun levels altered cell numbers, thus promoting regeneration. In aging mice, the results appear to exclude this, since cell numbers in the mutant and the WT are similar. During chronic denervation, Schwann cell numbers remain constant in c-Jun OE/+ mice, but fall by about 30% in the WT. Since there is now evidence that Schwann cell proliferation may not be essential for regeneration, contrary to common assumptions, the relationship between cell numbers and repair is currently unclear (Kim et al. 2000; Atanasoski et al. 2001; Yang et al. 2008; for discussion see Jessen and Mirsky 2019). Even in WT mice, cell number after chronic denervation remains nearly twice that in uninjured nerves. It is therefore unlikely that the changes in Schwann cell numbers are the key reason for the reduced regeneration support provided by 10-week cut WT stumps, or the increase in support provided by 10 week cut c-Jun OE/+ stumps.

The degree of reduction in transverse nerve area after chronic denervation could also affect repair. However, the area of 10 week cut c-Jun OE/+ nerves, while increased compared to 10 week cut WT, remains ~50% smaller than that of 2-week cut WT nerves. Nevertheless, regeneration through these nerves is similar. The relationship between nerve area and regeneration in these experiments may therefore not be straightforward.

During longer denervation times, cell loss and nerve shrinking will increasingly impede repair. These slow, atrophic changes, likely involving regulation of cell death and proliferation, have not been extensively studied, although STAT3 has recently been implicated in the long term maintenance of repair cells (Benito et al. 2017). Previously, c-Jun was shown to influence both apoptosis and proliferation in repair Schwann cells (Parkinson et al. 2001; 2004; 2008), but the particular way in which c-Jun levels determine nerve atrophy remains to be determined.

It has become clear that the injury-induced reprogramming of Schwann cells to cells specialized to support nerve regeneration is regulated by dedicated mechanisms, including c-Jun, STAT3, merlin and H3K27 trimethylation-related epigenetic controls, that operate selectively in repair cells, and have a relatively minor or undetectable function in Schwann

cell development (reviewed in Jessen and Mirsky 2019). The present work provides evidence that an impairment of one of these mechanisms, c-Jun, is a major contributor to two major categories of regeneration failure, aging and chronic denervation. It will be important to extend this study to other regulators of repair cells as a basis for developing molecular interventions for promoting repair in the PNS.

METHODS

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
<i>Mpz</i> <Cre/+>; <i>Rosa26c-Junstopf</i> <f/+>, C57BL/6J background, (Mus musculus, both sexes used)	c-Jun OE/+ mouse	Fazal et al. (2017)		RRID:MGI:
<i>Mpz</i> <Cre/+>; <i>Jun</i> <f/+>, C57BL/6J background, (Mus musculus, both sexes used)	c-Jun cKO mouse	Arthur-Farraj et al. (2012)	<i>Jun</i> ^{tm4Wag}	RRID:MGI:2445420
<i>Mpz</i> <Cre/+>; <i>Shh</i> <f/+>, C57BL/6J background, (Mus musculus, both sexes used)	Shh cKO mouse	Jackson Laboratory	B6;129- <i>Shh</i> ^{tm2Amc} /J	RRID:IMSR_JAX:004293

used)				
<i>Mpz</i> <Cre/+>, C57BL/6J background, (Mus musculus, both sexes used)	Mpz-Cre mouse	Jackson Laboratory	B6N.FVB-Tg(<i>Mpz-cre</i>)26Mes/J;	RRID:IMSR_JA X:017927
antibody	anti-c-Jun (rabbit monoclonal)	Cell Signaling	Cat #9165; RRID:AB_213 0165	WB (1:1000) IF (1:800)
antibody	anti-P-c-Jun (rabbit polyclonal)	Cell Signaling	Cat#9261; RRID:AB_213 0162	WB (1:1000)
antibody	anti-p75NTR (Ngfr) (rabbit polyclonal)	Millipore	Cat#AB1554; RRID:AB_907 60	WB (1:1000)
antibody	anti-GAPDH (rabbit polyclonal)	Sigma-Aldrich	Cat#G9545; RRID:AB_796 208	WB (1:5000)
antibody	anti-Canelxin (rabbit polyclonal)	Enzo Life Sciences	Cat#ADI-SPA-860-D; RRID:AB_2038 898	WB (1:1000)
antibody	anti-sox10 (goat polyclonal)	R&D Systems	Cat#AF2864; RRID:AB_442 208	IF (1:100)
antibody	anti-CGRP (rabbit monoclonal)	Peninsula Laboratories	Cat#T-4032; RRID:AB_518 147	IF (1:1000)
antibody	anti-Neurofilament (chicken polyclonal)	Abcam	Cat#ab4680; RRID:AB_304 560	IF (1:1000)

antibody	anti- Rabbit IgG, HRP-linked (Goat polyclonal)	Cell Signaling	Cat#7074; RRID:AB_209 9233	WB (1:2000)
antibody	Cy3 anti-Rabbit IgG (H+L) (Donkey polyclonal)	Jackson Immuno Research Labs	Cat#711-165-152; RRID:AB_230 7443	IF (1:500)
antibody	anti-Goat Alexa 488 Conjugated (Donkey polyclonal)	Molecular Probes - Thermo Fisher	Cat#A11057; RRID:AB_253 4104	IF (1:1000)
antibody	anti-Rabbit Alexa 488 Conjugated (Donkey polyclonal)	Molecular Probes - Thermo Fisher	Cat#A11008; RRID:AB_143 165	IF (1:1000)
antibody	anti-Chicken Alexa 488 Conjugated (Goat polyclonal)	Molecular Probes - Thermo Fisher	Cat#A-11039; RRID:AB_253 4096	IF (1:1000)
sequence-based reagent	<i>Bdnf_F</i>	Benito et al. (2017)	PCR primers	TCATACTTCG GTTGCATGAA GG
sequence-based reagent	<i>Bdnf_R</i>	Benito et al. (2017)	PCR primers	AGACCTCTCG AACCTGCCC
sequence-based reagent	<i>c-Jun_F</i> (Cells)	Benito et al. (2017)	PCR primers	AATGGGCACA TCACCACTAC AC
sequence-based reagent	<i>c-Jun_R</i> (Cells)	Benito et al. (2017)	PCR primers	TGCTCGTCGG TCACGTTCT

sequence-based reagent	<i>c-Jun_F</i> (Tissue)	Benito et al. (2017)	PCR primers	CCTTCTACGA CGATGCCCTC
sequence-based reagent	<i>c-Jun_R</i> (Tissue)	Benito et al. (2017)	PCR primers	GATTCGGGCC ACTTGGAGTT
sequence-based reagent	<i>Gdnf_F</i>	Benito et al. (2017)	PCR primers	GATTCGGGCC ACTTGGAGTT
sequence-based reagent	<i>Gdnf_R</i>	Benito et al. (2017)	PCR primers	GACAGCCACG ACATCCCATA
sequence-based reagent	<i>Calnexin_F</i>	Benito et al. (2017)	PCR primers	CAACAGGGGA GGTTTATTTG CT
sequence-based reagent	<i>Calnexin_R</i>	Benito et al. (2017)	PCR primers	TCCCACTTTC CATCATATTTG GC
sequence-based reagent	<i>Gapdh_F</i>	Benito et al. (2017)	PCR primers	AGGTCGGTGT GAACGGATTT G
sequence-based reagent	<i>Gapdh_R</i>	Benito et al. (2017)	PCR primers	TGTAGACCAT GTAGTTGAGG TCA
sequence-based reagent	<i>Mpz_F</i>	Benito et al. (2017)	PCR primers	GCTGGCCCAA ATGTTGCTGG
sequence-based reagent	<i>Mpz_R</i>	Benito et al. (2017)	PCR primers	CCACCACCTC TCCATTGCAC
commercial assay or kit	Kapa mRNA HyperPrep	Roche	Cat#KK8581, 08098123702	

	Kit			
commercial assay or kit	RNeasy Micro Extraction Kit	Qiagen	Cat#74004	
chemical compound, drug	Purmorphamine	Sigma-Aldrich	Cat#540220	Concentration: various, see figures
chemical compound, drug	Smoothed Agonist (SAG)	Merck-Sigma-Aldrich-Calbiochem	Cat#566660	Concentration: various, see figures
chemical compound, drug	Cyclopamine	Merck-Sigma-Aldrich-Calbiochem	Cat#CAS 4449-51-8	Concentration: various, see figures
software, algorithm	Samtools version 1.2	Li et al (2009)	RRID:SCR_002105	
software, algorithm	Picard tools version 1.140	http://broadinstitute.github.io/picard/	RRID:SCR_006525	
software, algorithm	featureCounts	Liao et al (2014)	RRID:SCR_012919	
software, algorithm	edgeR	Robinson et al (2010)	RRID:SCR_012802	
software, algorithm	Gene set enrichment analysis (GSEA)	Subramanian et al (2005)	RRID:SCR_003199	
software, algorithm	Gene ontology (GO) analysis – PANTHER classification system	Mi et al (2013)	RRID:SCR_004869	

software, algorithm	GraphPad Prism 9.0.0	GraphPad Prism	RRID:SCR_002 798	
software, algorithm	Bio Rad ChemiDoc MP Imaging System	Bio Rad	RRID:SCR_019 037	
other	Fluorogold	Fluorochro me	Fluoro-gold 20mg	Made up to 4%
other	DAPI	Thermo Fisher	Cat#D1306	IF (1:40000)

Transgenic mice

Animal experiments conformed to UK Home Office guidelines under the supervision of University College London (UCL) Biological Services under Protocol No. PPL/70/7900. Mice were generated to overexpress c-Jun selectively in Schwann cells as described (Fazal et al. 2017). Briefly, female *R26c-Junstopf* mice carrying a lox-P flanked STOP cassette in front of a CAG promoter-driven c-Jun cDNA in the ROSA26 locus, were crossed with male *Mpz^{Cre+/-}* mice (Feltri et al. 1999). This generated *Mpz^{Cre+};R26c-Junstop^{ff/+}*

mice, referred to here as c-Jun OE/+ mice. *Mpz^{Cre-/-}* littermates were used as controls. *Shh-floxed* mice, referred to as Shh cKO mice, carrying *loxP* sites flanking exon 2 of the *Shh* gene were obtained from the Jackson Laboratory (Jax®, stock# 004293), and bred to *Mpz^{Cre}* mice (Feltri et al., 1999). Experiments used mice of either sex on the C57BL/6 background.

Genotyping

DNA was extracted from ear notches or tail tips using the Hot Sodium Hydroxide and Tris method (HotSHOT; (Truett et al. 2000). Tissue was incubated in HotSHOT buffer (25 mM

NaOH and 0.2 mM disodium EDTA, pH 12) at 95°C for 1h. The reaction was neutralized with neutralizing buffer (40 mM Tris-HCl, pH 5). DNA was then added to the PCR mastermix with primers for the *Mpz-Cre* transgene: 5'-GCTGGCCCAAATGTTGCTGG-3' and 5'CCACCACCTCTCCATTGCAC-3' (480 bp band).

Surgery

For short-term time points (<1 week) and crushes, the sciatic nerve was exposed and cut or crushed (at 3 rotation angles) at the sciatic notch. For Western blot, immunofluorescence and mRNA investigations into chronic denervation (>1 week) the sciatic nerve was cut and the proximal stump was reflected back and sutured into muscle to prevent regeneration. The nerve distal to the injury was excised for analysis at various time points. Contralateral uninjured sciatic nerves served as controls. To examine the effects of chronic denervation, the nerve branches of the sciatic nerve were individually separated. (Fig. 2, Figure Supplement 1). The tibial nerve was cut and both proximal and distal stumps were reflected and sutured into muscle. Either immediately or following 10 weeks of chronic denervation, the distal tibial nerve stump was cut from the muscle and sutured to the freshly transected common peroneal nerve

Retrograde labelling with Fluorogold

To examine regeneration, following nerve crush or repair, the nerve was cut distal to the original injury site and exposed to 4% Fluorogold for 1 hour (Catapano et al. 2016; Fig. 1, Figure Supplement 1). The spinal cord and L4 DRG were removed following perfusion 1 week post-labeling. Labeled cells in all of the spinal cord sections (50µm) were counted and the Abercrombie correction was applied to compensate for double counting (Abercrombie 1946). To avoid double counting, cells in every fifth DRG section (20µm) were counted.

Schwann cell cultures

Rat Schwann cells were cultured as described (Brockes et al. 1979). Briefly, sciatic nerves and brachial plexuses were digested enzymatically with collagenase and trypsin and

cultured on laminin and PLL coated plates in DMEM, 2% FBS, 10ng/ml NRG-1, 2 μ M forskolin and penicillin/streptomycin. Under experimental conditions, cultures were maintained in defined medium (DMEM and Ham's F12 (1:1), transferrin (100 pg/ml), progesterone (60 ng/ml), putrescine (16 pg/ml), insulin (5 μ g/ml), thyroxine (0.4 μ g/ml), selenium (160 ng/ml), triiodothyronine (10.1 ng/ml), dexamethasone (38 ng/ml), glucose (7.9 mg/ml), bovine serum albumin (0.3 mg/ml), penicillin (100 IU/ml), streptomycin (100 IU/ml), and glutamine (2 mM) with 0.5% serum (Jessen et al., 1994; Meier et al. 1999).

Antibodies

Immunofluorescence antibodies: c-Jun (Cell Signaling Technology, rabbit 1:800), Sox10 (R&D Systems, goat 1:100), CGRP (Peninsula, rabbit 1:1000), Neurofilament (Abcam, rabbit 1:1000) donkey anti-goat IgG (H+L) Alexa Fluor 488 conjugate (Invitrogen, 1:1000), Cy3 donkey anti-rabbit IgG (H+L) (Jackson Immunoresearch, 1:500).

Antibodies used for Western blotting: c-Jun (Cell Signaling Technology, rabbit 1:1000), p75 NTR (Millipore, rabbit 1:1000), serine 63 phosphorylated c-Jun (Cell Signaling Technology, rabbit 1:1000), GAPDH (Sigma-Aldrich, rabbit 1:5000), calnexin (Enzo Life Sciences, rabbit 1:1000), anti-rabbit IgG, HRP-linked (Cell Signaling Technology, 1:2000).

Immunofluorescence

For immunofluorescence experiments on cultured cells, 5000 Schwann cells were plated in a 35 μ l drop on a PLL laminin coated coverslip. Cells were topped up with defined medium after 24 hours. At the experimental end point, cells were washed 2x with 1x PBS. Cells were fixed with 4% PFA for 10 minutes. Cells were then washed for 5 minutes in 1x PBS. Fresh PBS was added to the wells and the lid was parafilm sealed. Dishes were stored at 4°C until use.

Nerve samples were fresh frozen during embedding in OCT. Cryosections were cut at 10 μ m. Sections were fixed in 100% acetone (Sox10/c-Jun double-labeling, 10 minutes at -20°C) or 4% PFA (10 minutes at room temperature).

For immunofluorescence, all samples were washed 3x in 1x PBS and blocked in 5% donkey serum, 1% BSA, 0.3% Triton X-100 in PBS. Samples were incubated with primary antibodies in blocking solution overnight at 4°C. Sox10/c-Jun double-labeling was performed overnight at room temperature. Samples were washed and incubated with secondary antibodies and DAPI to identify cell nuclei (Thermo Fisher Scientific, 1:40,000) in PBS for 1 hour at room temperature. Samples were mounted in fluorescent mounting medium (Citifluor).

Images were taken on a Nikon Labophot 2 fluorescence microscope. Cell counts were performed in ImageJ or directly from the microscope. Comparable images have been equally adjusted for brightness/contrast. In some cases (Figs 1,3 and 4), images of whole nerve profiles have been generated by stitching together multiple images.

Western Blotting

Nerves were dissected and snap frozen in liquid nitrogen. For protein extraction, nerves were placed in 2ml graduated skirted tubes with 9 10B lysing beads with 75ml lysis buffer (1M Tris-HCl pH 8, 5M NaCl, 20% Triton X-100, 5mM EDTA) and homogenized using a Fastprep fp120 homogeniser. Samples were run twice at speed 6 for 45 seconds. Lysates were then centrifuged at 13000rpm for 2 minutes at 4°C to pellet the debris. The supernatant was transferred to a new 1.5ml Eppendorf tube and centrifuged at 13000rpm for 2 minutes at 4°C. The supernatant was transferred to a new 1.5ml Eppendorf tube and the protein extract was stored at -80°C.

For protein studies on cultured cells, 1×10^6 purified Schwann cells were plated in a 35mm dish in defined medium for 48 hours. At the time of extraction, the cultures were washed 2x with 1x PBS and incubated with 100µl cell lysis buffer (T-PER Tissue Protein Extraction Reagent, Halt protease & phosphatase inhibitor cocktail (1:100) Thermo Fisher Scientific). Cells were physically detached from dishes using a cell scraper. The cell lysate was collected and kept on ice in a 1.5ml Eppendorf tube. Lysate was spun for 2 minutes at 1000rpm to pellet the debris. The supernatant was transferred to a fresh Eppendorf tube and

642 spun for a further 2 minutes at 1000rpm. The supernatant was transferred to a new 1.5ml
643 Eppendorf tube and stored at -80°C until use.

644 Protein was diluted in the appropriate lysis buffer and 5x Laemmli buffer at a working
645 concentration of 1x. Samples were heated to 95°C for 5 minutes to denature the protein.
646 10µg protein was loaded per well on 8% acrylamide gels with prestained standard molecular
647 weight markers (PageRuler prestained protein ladder; Thermo Fisher Scientific) and run at
648 60mV for 3 hours using the mini Protean II gel electrophoresis apparatus (Bio-Rad
649 Laboratories). Protein was transferred to a nitrocellulose membrane (Hybond ECL;
650 Amersham Biosciences) using a semi-dry transfer system (Bio-Rad Laboratories) at 25mV
651 for 45 minutes. Membranes were briefly stained with Ponceau S (Sigma Aldrich) to
652 determine that the transfer has been successful and that equal levels of protein had been
653 loaded in the gel. Membranes were briefly washed in ddH₂O to remove excess Ponceau and
654 blocked in 5% milk/TBS-T for 1 hour with shaking at room temperature. Membranes were
655 then incubated with appropriate antibodies in heat sealable polyethylene bags and were
656 incubated overnight at 4°C on a rotatory wheel. Membranes were washed 3x for 10 minutes
657 in 1x TBS-T then incubated with the appropriate secondary antibody in heat sealable
658 polyethylene bags, rotating for 1 hour at room temperature. Membranes were washed 3x for
659 10 minutes in 1x TBS-T before developing. For development of GAPDH, membranes were
660 incubated with ECL (Amersham) for 1 minute and developed on a Bio-Rad Chemidoc
661 machine. For the development of all other antibodies, membranes were incubated with ECL
662 prime (Amersham) for 5 minutes then developed. Membranes were automatically exposed to
663 prevent saturation. Blots were analysed and densitometric quantification was performed
664 using Bio-Rad Imagelab. Protein levels were determined by normalising the protein of
665 interest against the house keeping protein (GAPDH or calnexin). All blots were then
666 normalized to one sample (e.g 1 week after injury, control cells) to account for any difference
667 between each blot. Each experiment was performed at least 3 times with fresh samples.
668 Representative images are shown.

Electron microscopy

Nerves were fixed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4°C. Post-fixation in 1% OsO₄ was performed before nerves were embedded in Agar 100 epoxy resin. Transverse ultrathin sections from adult (P60) or aged (P300) tibial nerves or from injured distal stumps of adult sciatic nerves at various times after injury were taken 5mm from the sciatic notch and mounted on film (no grid bars). Images were examined using a Jeol 1010 electron microscope with a Gatan camera and software. Images were examined and photographed at 8000x or 15,000x. The nerve area was measured from photographs taken at 200x magnification. Schwann cells and macrophages and fibroblasts were identified by standard ultrastructural criteria (e.g. Reichert et al. 1994). Schwann cell, macrophage and fibroblast nuclei were counted in every field, or every second, third or fourth field, depending on the size of the nerve, and multiplied by the number of fields to generate totals.

qPCR

RNA from rat Schwann cell cultures or mouse nerve tissue was extracted using an RNeasy Micro Extraction Kit (Qiagen). RNA quality and concentration was determined after extraction using a nanodrop 2000 machine (Thermo). 1µg of RNA was converted to cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. Samples were run with PrecisionPLUS qPCR Mastermix with SYBR Green (Primerdesign) with primers as described in Benito et al. (2017). Ct values were normalized to housekeeping gene expression (*GAPDH* and *calnexin*).

Primer	Sequence 5'-3'
<i>Bdnf</i> Fwd	TCATACTTCGGTTGCATGAAGG
<i>Bdnf</i> Rev	AGACCTCTCGAACCTGCCC
<i>c-Jun</i> Fwd (Cells)	AATGGGCACATCACCCTACAC

<i>c-Jun</i> Rev (Cells)	TGCTCGTCGGTCACGTTCT
<i>c-Jun</i> Fwd (Tissue)	CCTTCTACGACGATGCCCTC
<i>c-Jun</i> Rev (Tissue)	GGTTCAAGGTCATGCTCTGTTT
<i>Gdnf</i> Fwd	GATTCGGGCCACTTGGAGTT
<i>Gdnf</i> Rev	GACAGCCACGACATCCCATA
<i>Calnexin</i> Fwd	CAACAGGGGAGGTTTATTTTGCT
<i>Calnexin</i> Rev	TCCCACTTTCCATCATATTTGGC
<i>GAPDH</i> Fwd	AGGTCGGTGTGAACGGATTG
<i>GAPDH</i> Rev	TGTAGACCATGTAGTTGAGGTCA

690

691 *Statistical analysis*

692 Results are expressed as mean \pm SEM. Statistical significance was estimated by
693 Student's *t* test, one-way ANOVA or two-way ANOVA with appropriate post hoc tests.
694 A *p* value <0.05 was considered as statistically significant. Statistical analysis was performed
695 using GraphPad software.

696 *Library preparation*

697 RNA was extracted using a RNeasy lipid tissue kit with an in column DNase step (Qiagen).
698 Chronically denervated and uninjured nerves were pooled, 2 per n. Acutely denervated
699 nerves were not pooled using 1 nerve per n. The library was prepared using the Kapa mRNA
700 Hyper Prep kit (Roche) with 100ng RNA and 15 cycles of PCR enrichment. The assay is
701 (first) stranded (dUTP method).

702 *Sequencing*

703 Sequencing was performed in a pooled NextSeq 500 run using a 43bp paired end protocol
704 (plus a 6bp index read). Sequencing reads (in fastq format) were aligned to the hg38
705 reference sequence using STAR v2.5.3 (Dobin et al. 2013). Samtools version 1.2 and Picard

tools version 1.140 were used to process alignments (Li et al. 2009)' Aligned reads were filtered for mapq ≥ 4 i.e. uniquely mapping reads, and putative PCR duplicates were removed. featureCounts was used to perform read summarization (Liao et al. 2014).

Data Analysis

Expression analysis was carried out using R version 3.5.1. Differential gene expression was analysed using edgeR (Robinson et al. 2010). Genes with both an absolute log₂ fold change > 2.0 and FDR < 0.05 were identified as being significantly differentially expressed. Principal Component Analysis (PCA) showed that injury status was the dominant source of variation in both data sets (Fig. 7 Figure Supplement 1 A; Fig. 8 Figure Supplement 1A). Enrichment of c-Jun regulated genes was investigated using Fisher one-sided exact tests and gene set enrichment analysis (GSEA; Subramanian et al. 2005). Gene ontology (GO) analysis was used to examine gene enrichment of all significantly differentiated genes using the PANTHER classification system (Mi et al. 2013).

721

722

REFERENCES

723

724 Abercrombie, M. 1946. Estimation of nuclear population from microtome sections. The
725 Anatomical record 94, pp. 239–247.

726 Allodi, I., Udina, E. and Navarro, X. 2012. Specificity of peripheral nerve regeneration:
727 interactions at the axon level. Progress in Neurobiology 98, pp.16–37.

728 Arthur-Farraj, P.J., Morgan, C.C., Adamowicz, M., Gomez-Sanchez, J.A., Fazal, S.V.,
729 Beucher, A., Razzaghi, B., Mirsky, R., Jessen, K.R., Aitman, T.J. (2017). Changes in the
730 Coding and Non-coding Transcriptome and DNA Methylome That Define the Schwann Cell
731 Repair Phenotype After Nerve Injury. Cell Reports 20(11), pp. 2719-2734.

732 Arthur-Farraj, P.J., Latouche, M., Wilton, D.K., Quintes, S., Chabrol, E., Banerjee, A.,
733 Woodhoo, A., Jenkins, B., Rahman, M., Turmaine, M., Wicher, G.K., Mitter, R., Greensmith,
734 L., Behrens, A., Raivich, G., Mirsky, R. and Jessen, K.R. 2012. c-Jun reprograms Schwann
735 cells of injured nerves to generate a repair cell essential for regeneration. Neuron 75(4), pp.
736 633–647.

737 Atanasoski, S., Shumas, S., Dickson, C., Scherer, S.S. and Suter, U. 2001. Differential
738 cyclin D1 requirements of proliferating Schwann cells during development and after injury.
739 Molecular and Cellular Neurosciences 18(6), pp. 581–592.

740 Benito, C., Davis, C.M., Gomez-Sanchez, J.A., Turmaine, M., Meijer, D., Poli, V., Mirsky, R.
741 and Jessen, K.R. 2017. STAT3 Controls the Long-Term Survival and Phenotype of Repair
742 Schwann Cells during Nerve Regeneration. The Journal of Neuroscience 37(16), pp. 4255–
743 4269.

744 Boerboom, A., Dion, V., Chariot, A. and Franzen, R. 2017. Molecular mechanisms involved
745 in schwann cell plasticity. Frontiers in Molecular Neuroscience 10, p. 38.

746 Bond, C.W., Angeloni, N., Harrington, D., Stupp, S. and Podlasek, C.A. 2013. Sonic
747 Hedgehog regulates brain-derived neurotrophic factor in normal and regenerating cavernous
748 nerves. The Journal of Sexual Medicine 10(3), pp. 730–737.

749 Boyd, J. G., and Gordon, T. (2003). Neurotrophic factors and their receptors in axonal
750 regeneration and functional recovery after peripheral nerve injury. Mol. Neurobiol. 27, pp.
751 277–324.

752 Boyd, J.G. and Gordon, T. 2003. Glial cell line-derived neurotrophic factor and brain-derived
 753 neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons
 754 in vivo. *Experimental Neurology* 183(2), pp. 610–619.

755 Brockes, J.P., Fields, K.L. and Raff, M.C. 1979. Studies on cultured rat Schwann cells. I.
 756 Establishment of purified populations from cultures of peripheral nerve. *Brain Research*
 757 165(1), pp. 105–118.

758 Brosius Lutz, A., Chung, W.-S., Sloan, S.A., Carson, G.A., Zhou, L., Lovelett, E., Posada, S.,
 759 Zuchero, J.B. and Barres, B.A. 2017. Schwann cells use TAM receptor-mediated
 760 phagocytosis in addition to autophagy to clear myelin in a mouse model of nerve injury.
 761 *Proceedings of the National Academy of Sciences of the United States of America* 114(38),
 762 pp. E8072–E8080.

763 Brushart, T. M., Aspalter, M., Griffin, J. W., Redett, R., Hameed, H., Zhou, C., Wright M,
 764 Vyas A, Höke A. 2013. Schwann cell phenotype is regulated by axon modality and central-
 765 peripheral location and persists in vitro. *Experimental Neurology* 247, pp. 272–281.

766 Büttner, R., Schulz, A., Reuter, M., Akula, A.K., Mindos, T., Carlstedt, A., Riecken, L.B.,
 767 Baader, S.L., Bauer, R. and Morrison, H. 2018. Inflammaging impairs peripheral nerve
 768 maintenance and regeneration. *Aging Cell* 17(6), p. e12833.

769 Catapano, J., Willand, M.P., Zhang, J.J., Scholl, D., Gordon, T. and Borschel, G.H. 2016.
 770 Retrograde labeling of regenerating motor and sensory neurons using silicone caps. *Journal*
 771 *of Neuroscience Methods* 259, pp. 122–128.

772 Chen, Z.L., Yu, W.M. and Strickland, S. 2007. Peripheral regeneration. *Annual Review of*
 773 *Neuroscience*. 30, pp. 209-33.

774 Chung, W.-S., Clarke, L.E., Wang, G.X., Stafford, B.K., Sher, A., Chakraborty, C., Joung, J.,
 775 Foo, L.C., Thompson, A., Chen, C., Smith, S.J. and Barres, B.A. 2013. Astrocytes mediate
 776 synapse elimination through MEGF10 and MERTK pathways. *Nature* 504(7480), pp. 394–
 777 400.

778 Clements, M.P., Byrne, E., Camarillo Guerra, L.F., Cattin, A., Zakka, L., Ashraf, A., Burden,
 779 J.J., Khadayate, S., Lloyd, A.C., Marguerat, S., Parrinello, S. 2017. The Wound
 780 Microenvironment Reprograms Schwann Cells to Invasive Mesenchymal-like Cells to Drive
 781 Peripheral Nerve Regeneration. *Neuron* 96(1), pp 98-114.

782 Cohen, D.E. and Melton, D. 2011. Turning straw into gold: directing cell fate for regenerative
 783 medicine. *Nature Review in Genetics* 12, pp. 243-252.

784 Dobin, A. et al., 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1),
785 pp.15–21.

786 Doron-Mandel, E., Fainzilber, M. and Terenzio, M. 2015. Growth control mechanisms in
787 neuronal regeneration. *FEBS Letters* 589, pp.1669–1677.

788 Eggers, R., Tannemaat, M.R., Ehlert, E.M. and Verhaagen, J. 2010. A spatio-temporal
789 analysis of motoneuron survival, axonal regeneration and neurotrophic factor expression
790 after lumbar ventral root avulsion and implantation. *Experimental Neurology* 223(1), pp. 207–
791 220.

792 Eguizabal, C., Montserrat, N., Veiga A. and Izpisua Belmonte, J.C. 2013. Dedifferentiation,
793 transdifferentiation, and reprogramming: future directions in regenerative medicine.
794 *Seminars in Reproductive Medicine* 31, pp. 82-94.

795 Fawcett, J.W. and Verhaagen, J. 2018. Intrinsic Determinants of Axon Regeneration.
796 *Developmental Neurobiology* 78(10), pp. 890-897.

797 Fazal, S.V., Gomez-Sanchez, J.A., Wagstaff, L.J., Musner, N., Otto, G., Janz, M., Mirsky, R.
798 and Jessen, K.R. 2017. Graded Elevation of c-Jun in Schwann Cells In Vivo: Gene Dosage
799 Determines Effects on Development, Remyelination, Tumorigenesis, and Hypomyelination.
800 *The Journal of Neuroscience* 37(50), pp. 12297–12313.

801 Feltri, M.L., D'Antonio, M., Previtali, S., Fasolini, M., Messing, A. and Wrabetz, L. 1999. P0-
802 Cre transgenic mice for inactivation of adhesion molecules in Schwann cells. *Annals of the*
803 *New York Academy of Sciences* 883, pp. 116–123.

804 Fontana, X., Hristova, M., Da Costa, C., Patodia, S., Thei, L., Makwana, M., Spencer-Dene,
805 B., Latouche, M., Mirsky, R., Jessen, K.R., Klein, R., Raivich, G., Behrens, A. 2012. c-Jun in
806 Schwann cells promotes axonal regeneration and motoneuron survival via paracrine
807 signalling. *Journal of Cell Biology* 198(1), pp. 127–141.

808 Fu, S.Y. and Gordon, T. 1995. Contributing factors to poor functional recovery after delayed
809 nerve repair: prolonged denervation. *The Journal of Neuroscience* 15(5 Pt 2), pp. 3886–
810 3895.

811 Furey, M.J., Midha, R., Xu, Q.-G., Belkas, J. and Gordon, T. 2007. Prolonged target
812 deprivation reduces the capacity of injured motoneurons to regenerate. *Neurosurgery* 60(4),
813 pp. 723–732.

814 Gambarotta, G., Fregnan, F., Gnani, S. and Perroteau, I. 2013. Neuregulin 1 role in
815 Schwann cell regulation and potential applications to promote peripheral nerve regeneration.
816 *International Review of Neurobiology* 108, pp. 223-256.

817 Glenn, T. D. and Talbot, W. S. 2013. Signals regulating myelination in peripheral nerves and
818 the Schwann cell response to injury. *Current Opinion in Neurobiology* 23, pp. 1041–1048.

819 Gomez-Sanchez, J.A., Pilch, K.S., van der Lans, M., Fazal, S.V., Benito, C., Wagstaff, L.J.,
820 Mirsky, R. and Jessen, K.R. 2017. After nerve injury, lineage tracing shows that myelin and
821 Remak Schwann cells elongate extensively and branch to form repair schwann cells, which
822 shorten radically on remyelination. *The Journal of Neuroscience* 37(37), pp. 9086–9099.

823 Graciarena, M., Dambly-Chaudière, C. and Ghysen, A. 2014. Dynamics of axonal
824 regeneration in adult and aging zebrafish reveal the promoting effect of a first lesion.
825 *Proceedings of the National Academy of Sciences of the United States of America* 111(4),
826 pp. 1610–1615.

827 Grothe, C., Haastert, K. and Jungnickel, J. 2006. Physiological function and putative
828 therapeutic impact of the FGF-2 system in peripheral nerve regeneration—lessons from in
829 vivo studies in mice and rats. *Brain Research Reviews* 51, pp. 293–299.

830 Hashimoto, M., Ishii, K., Nakamura, Y., Watabe, K., Kohsaka, S. and Akazawa, C. 2008.
831 Neuroprotective effect of sonic hedgehog up-regulated in Schwann cells following sciatic
832 nerve injury. *Journal of Neurochemistry* 107(4), pp. 918–927.

833 Hiraiwa, M., Campana, W.M., Mizisin, A.P., Mohiuddin, L. and O'Brien, J.S. 1999.
834 Prosaposin: a myelinotrophic protein that promotes expression of myelin constituents and is
835 secreted after nerve injury. *Glia* 26(4), pp. 353–360.

836 Hiraiwa, M., Taylor, E.M., Campana, W.M., Darin, S.J. and O'Brien, J.S. 1997. Cell death
837 prevention, mitogen-activated protein kinase stimulation, and increased sulfatide
838 concentrations in Schwann cells and oligodendrocytes by prosaposin and prosaptides.
839 *Proceedings of the National Academy of Sciences of the United States of America* 94(9), pp.
840 4778–4781.

841 Höke, A. 2006. Mechanisms of disease: what factors limit the success of peripheral nerve
842 regeneration in humans? *Nature Clinical Practice Neurology* 2, 448–454.

843 Höke, A., Gordon, T., Zochodne, D.W. and Sulaiman, O.A.R. 2002. A decline in glial cell-
844 line-derived neurotrophic factor expression is associated with impaired regeneration after
845 long-term Schwann cell denervation. *Experimental Neurology* 173(1), pp. 77–85.

846 Hsin-Pin, L., Oksuz, I., Hurley, E., Wrabetz, L., Awatramani, R., 2015. Microprocessor
847 Complex Subunit DiGeorge Syndrome Critical Region Gene 8 (Dgcr8) Is Required for
848 Schwann Cell Myelination and Myelin Maintenance. *Journal of Biological Chemistry*, 290,
849 pp.24294-24307.

850 Huang, L., Quan, X., Liu, Z., Ma, T., Wu, Y., Ge, J., Zhu, S., Yang, Y., Liu, L., Sun, Z.,
851 Huang, J. and Luo, Z. 2015. c-Jun gene-modified Schwann cells: upregulating multiple
852 neurotrophic factors and promoting neurite outgrowth. *Tissue Engineering. Part A* 21(7-8),
853 pp. 1409–1421.

854 Huang, L., Xia, B., Shi, X., Gao, J., Yang, Y., Xu, F., Qi, F., Liang, C., Huang, J. and Luo, Z.
855 2019. Time-restricted release of multiple neurotrophic factors promotes axonal regeneration
856 and functional recovery after peripheral nerve injury. *The FASEB Journal*, 33(7), pp.8600-
857 8613.

858 Jessen, K.R. and Arthur-Farraj, P. 2019. Repair Schwann cell update: Adaptive
859 reprogramming, EMT, and stemness in regenerating nerves. *Glia* 67(3), pp. 421–437.

860 Jessen, K.R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y. and Gavrillovic, J.
861 1994. The Schwann cell precursor and its fate: a study of cell death and differentiation
862 during gliogenesis in rat embryonic nerves. *Neuron* 12(3), pp. 509–527.

863 Jessen, K.R. and Mirsky, R. 2016. The repair Schwann cell and its function in regenerating
864 nerves. *The Journal of Physiology* 594(13), pp. 3521–3531.

865 Jessen, K.R. and Mirsky, R. 2019. The success and failure of the schwann cell response to
866 nerve injury. *Frontiers in Cellular Neuroscience* 13, p. 33.

867 Jessen, K.R., Mirsky, R., Arthur-Farraj, P. 2015. The Role of Cell Plasticity in Tissue Repair:
868 Adaptive Cellular Reprogramming. *Developmental Cell* 34, pp. 613-620

869 Jonsson, S., Wiberg, R., McGrath, A.M., Novikov, L.N., Wiberg, M., Novikova, L.N. and
870 Kingham, P.J. 2013. Effect of delayed peripheral nerve repair on nerve regeneration,
871 Schwann cell function and target muscle recovery. *Plos One* 8(2), p. e56484.

872 Kim, H.A., Pomeroy, S.L., Whoriskey, W., Pawlitzky, I., Benowitz, L.I., Sicinski, P., Stiles,
873 C.D. and Roberts, T.M. 2000. A developmentally regulated switch directs regenerative
874 growth of Schwann cells through cyclin D1. *Neuron* 26(2), pp. 405–416.

875 Kudo, K., Gavin, E., Das, S., Amable, L., Shevde, L.A. and Reed, E. 2012. Inhibition of Gli1
876 results in altered c-Jun activation, inhibition of cisplatin-induced upregulation of ERCC1,

877 XPD and XRCC1, and inhibition of platinum–DNA adduct repair. *Oncogene*, 31(44),
878 pp.4718-4724.

879 Laner-Plamberger, S., Kaser, A., Paulischta, M., Hauser-Kronberger, C., Eichberger, T. and
880 Frischauf, A. M. 2009. Cooperation between GLI and JUN enhances transcription of JUN
881 and selected GLI target genes. *Oncogene* 28(13), pp.1639-1651.

882 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis,
883 G., Durbin, R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*,
884 25(16), pp.2078–2079.

885 Liao, Y., Smyth, G.K. & Shi, W., 2014. featureCounts: an efficient general purpose program
886 for assigning sequence reads to genomic features. *Bioinformatics*, 30(7), pp.923–930.

887 Lu, Q.R., Yuk, D., Alberta, J.A., Zhu, Z., Pawlitzky, I., Chan, J., McMahon, A.P., Stiles, C.D.,
888 Rowitch, D.H. 2000. Sonic hedgehog--regulated oligodendrocyte lineage genes encoding
889 bHLH proteins in the mammalian central nervous system. *Neuron*. 25, pp. 317-329.

890 Ma, K.H., Hung, H.A., Srinivasan, R., Xie, H., Orkin, S.H. and Svaren, J. 2015. Regulation of
891 Peripheral Nerve Myelin Maintenance by Gene Repression through Polycomb Repressive
892 Complex 2. *The Journal of Neuroscience* 35(22), pp. 8640–8652.

893 Ma, K.H., Hung, H.A. and Svaren, J. 2016. Epigenomic regulation of schwann cell
894 reprogramming in peripheral nerve injury. *The Journal of Neuroscience* 36(35), pp. 9135–
895 9147.

896 Martinez, J.A., Kobayashi, M., Krishnan, A., Webber, C., Christie, K., Guo, G., Singh, V. and
897 Zochodne, D.W. 2015. Intrinsic facilitation of adult peripheral nerve regeneration by the
898 Sonic hedgehog morphogen. *Experimental Neurology* 271, pp. 493–505.

899 Meier, C., Parmantier, E., Brennan, A., Mirsky, R. and Jessen, K.R. 1999. Developing
900 Schwann cells acquire the ability to survive without , axons by establishing an autocrine
901 circuit involving Insulin-like growth factor, neurotrophin-3, and Platelet-derived growth factor-
902 BB. *Journal of Neuroscience* 19(10), pp. 3847–3859.

903 Melcangi, R.C., Magnaghi, V., Cavarretta, I., Riva, M.A., Piva, F. and Martini, L. 1998.
904 Effects of steroid hormones on gene expression of glial markers in the central and peripheral
905 nervous system: variations induced by aging. *Experimental Gerontology* 33(7-8), pp 827-36.

906 Melcangi, R.C, Magnaghi, V. and Martini L. 2000. Aging in peripheral nerves: regulation of
907 myelin protein genes by steroid hormones. *Progress in Neurobiology* 60(3), pp. 291-308.

908 Meyer, R.C., Giddens, M.M., Schaefer, S.A. and Hall, R.A. 2013. GPR37 and GPR37L1 are
 909 receptors for the neuroprotective and glioprotective factors prosaptide and prosaposin.
 910 Proceedings of the National Academy of Sciences of the United States of America 110(23),
 911 pp. 9529–9534.

912 Mi, H., Muruganujan, A., Casagrande, J.T., and Thomas, P.D. 2013. Large-scale gene
 913 function analysis with the PANTHER classification system. Nature Protocols, 8(8), pp.1551–
 914 1566.

915 Michalski, B., Bain, J.R. and Fahnstock, M. 2008. Long-term changes in neurotrophic factor
 916 expression in distal nerve stump following denervation and reinnervation with motor or
 917 sensory nerve. Journal of Neurochemistry 105(4), pp. 1244–1252.

918 Nocera, G. and Jacob, C. 2020. Mechanisms of Schwann cell plasticity involved in
 919 peripheral nerve repair after injury. Cellular and Molecular Life Sciences 77(20):3977-3989.

920 Novikova, L., Novikov, L. and Kellerth, J.O. 1997. Persistent neuronal labeling by retrograde
 921 fluorescent tracers: a comparison between Fast Blue, Fluoro-Gold and various dextran
 922 conjugates. Journal of Neuroscience Methods 74(1), pp. 9–15.

923 Painter, M.W. 2017. Aging Schwann cells: mechanisms, implications, future directions.
 924 Current Opinion in Neurobiology 47, pp. 203–208.

925 Painter, M.W., Brosius Lutz, A., Cheng, Y.-C., Latremoliere, A., Duong, K., Miller, C.M.,
 926 Posada, S., Cobos, E.J., Zhang, A.X., Wagers, A.J., Havton, L.A., Barres, B., Omura, T. and
 927 Woolf, C.J. 2014. Diminished Schwann cell repair responses underlie age-associated
 928 impaired axonal regeneration. Neuron 83(2), pp. 331–343.

929 Parkinson, D.B., Bhaskaran, A., Arthur-Farraj, P., Noon, L.A., Woodhoo, A., Lloyd, A.C.,
 930 Feltri, M.L., Wrabetz, L., Behrens, A., Mirsky, R. and Jessen, K.R. 2008. c-Jun is a negative
 931 regulator of myelination. The Journal of Cell Biology 181(4), pp. 625–637.

932 Parkinson, D.B., Bhaskaran, A., Droggiti, A., Dickinson, S., D'Antonio, M., Mirsky, R. and
 933 Jessen, K.R. 2004. Krox-20 inhibits Jun-NH2-terminal kinase/c-Jun to control Schwann cell
 934 proliferation and death. The Journal of Cell Biology 164(3), pp. 385–394.

935 Parkinson, D.B., Dong, Z., Bunting, H., Whitfield, J., Meier, C., Marie, H., Mirsky, R. and
 936 Jessen, K.R., 2001. Transforming growth factor β (TGF β) mediates Schwann cell death in
 937 vitro and in vivo: examination of c-Jun activation, interactions with survival signals, and the
 938 relationship of TGF β -mediated death to Schwann cell differentiation. Journal of
 939 Neuroscience, 21(21), pp.8572-8585.

940 Pepinsky, R.B., Shapiro, R.I., Wang, S., Chakraborty, A., Gill, A., Lepage, D.J., Wen, D.,
 941 Rayhorn, P., Horan, G.S., Taylor, F.R. and Garber, E.A., 2002. Long-acting forms of sonic
 942 hedgehog with improved pharmacokinetic and pharmacodynamic properties are efficacious
 943 in a nerve injury model. *Journal of pharmaceutical sciences*, 91(2), pp.371-387.

944 Pestronk, A., Drachman, D.B. and Griffin, J.W. 1980. Effects of aging on nerve sprouting and
 945 regeneration. *Experimental Neurology* 70(1), pp. 65–82.

946 Reichert, F., Saada, A. and Rotshenker, S. 1994. Peripheral nerve injury induces Schwann
 947 cells to express two macrophage phenotypes: phagocytosis and galactose specific lectin
 948 MAC-2. *Journal of Neuroscience* 14, pp.3231-3245.

949 Robinson, M.D., McCarthy, D.J. & Smyth, G.K., 2010. edgeR: a Bioconductor package for
 950 differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1),
 951 pp.139–140.

952 Ruijs, A.C.J., Jaquet, J., Kalmijn, S., Giele, H., Hovius, S.E.R. 2005. Median and ulnar
 953 nerve injuries: A meta-analysis of predictors of motor and sensory recovery after modern
 954 microsurgical nerve repair. *Plastic Reconstructive Surgery* 116(2), pp. 484-494.

955 Scheib, J. and Höke A. 2013. Advances in peripheral nerve regeneration. *Nature Reviews*
 956 *Neurology* 9(12), pp.668-676.

957 Scheib, J.L. and Höke, A. 2016. An attenuated immune response by Schwann cells and
 958 macrophages inhibits nerve regeneration in aged rats. *Neurobiology of Aging* 45, pp. 1–9.

959 Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A.,
 960 Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. 2005. Gene set
 961 enrichment analysis: a knowledge-based approach for interpreting genome-wide expression
 962 profiles. *Proceedings of the National Academy of Sciences of the United States of America*,
 963 102(43), pp.15545–15550.

964 Sulaiman, O.A. and Gordon, T. 2000. Effects of short- and long-term Schwann cell
 965 denervation on peripheral nerve regeneration, myelination, and size. *Glia* 32(3), pp. 234–
 966 246.

967 Sulaiman, O. A. and Gordon, T. 2009. Role of chronic Schwann cell denervation in poor
 968 functional recovery after nerve injuries and experimental strategies to combat it.
 969 *Neurosurgery* 65, A105–A114.

970 Tanaka, K., Eskin, A., Chareyre, F., Jessen, W.J., Manent, J., Niwa-Kawakita, M., Chen, R.,
 971 White, C.H., Vitte, J., Jaffer, Z.M., Nelson, S.F., Rubenstein, A.E. and Giovannini, M. 2013.

972 Therapeutic potential of HSP90 inhibition for neurofibromatosis type 2. *Clinical Cancer*
973 *Research* 19(14), pp. 3856–3870.

974 Tanaka, K. and Webster, H.D. 1991. Myelinated fiber regeneration after crush injury is
975 retarded in sciatic nerves of aging mice. *The Journal of Comparative Neurology* 308(2), pp.
976 180–187.

977 Tanaka, K., Zhang, Q.L. and Webster, H.D. 1992. Myelinated fiber regeneration after sciatic
978 nerve crush: morphometric observations in young adult and aging mice and the effects of
979 macrophage suppression and conditioning lesions. *Experimental Neurology* 118(1), pp. 53–
980 61.

981 Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A. and Warman, M.L. 2000.
982 Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris
983 (HotSHOT). *Biotechniques* 29(1), pp. 52–54.

984 Vaughan, D.W. 1992. Effects of advancing age on peripheral nerve regeneration. *The*
985 *Journal of Comparative Neurology* 323(2), pp. 219–237.

986 Verdú, E., Ceballos, D., Vilches, J.J. and Navarro, X. 2000. Influence of aging on peripheral
987 nerve function and regeneration. *Journal of the Peripheral Nervous System* 5(4), pp. 191–
988 208.

989 Weiss, T., Taschner-Mandl, S., Bileck, A., Slany, A., Kromp, F., Rifatbegovic, F., Frech, C.,
990 Windhager, R., Kitzinger, H., Tzou, C.-H., Ambros, P.F., Gerner, C. and Ambros, I.M. 2016.
991 Proteomics and transcriptomics of peripheral nerve tissue and cells unravel new aspects of
992 the human Schwann cell repair phenotype. *Glia* 64(12), pp. 2133–2153.

993 Wilcox, M.B., Laranjeira, S.G., Eriksson, T.M., Jessen, K.R., Mirsky, R., Quick, T.J. and
994 Phillips, J.B. 2020. Characterising cellular and molecular features of human peripheral nerve
995 degeneration. *Acta Neuropathologica Communications* 8(1), p. 51.

996 Wolter, P., Schmitt, K., Fackler, M., Kremling, H., Probst, L., Hauser, S., Gruss, O.J. and
997 Gaubatz, S. 2012. GAS2L3, a target gene of the DREAM complex, is required for proper
998 cytokinesis and genomic stability. *Journal of Cell Science* 125(Pt 10), pp. 2393–2406.

999 Yamada, Y., Ohazama, A., Maeda, T., Seo, K. 2018. The Sonic Hedgehog signaling
1000 pathway regulates inferior alveolar nerve regeneration. *Neuroscience Letters* 671, pp 114–
1001 119.

Yamada, Y., Trakanant, S., Nihara, J., Kudo, T., Seo, K., Saeki, M., Kurose, M., Matsumaru, D., Maeda, T. and Ohazama, A. 2020. Gli3 is a key factor in the Schwann Cells from both intact and injured peripheral nerves. *Neuroscience* 432, pp. 229–239.

Yang, D.P., Zhang, D.P., Mak, K.S., Bonder, D.E., Pomeroy, S.L. and Kim, H.A. 2008. Schwann cell proliferation during Wallerian degeneration is not necessary for regeneration and remyelination of the peripheral nerves: axon-dependent removal of newly generated Schwann cells by apoptosis. *Molecular and Cellular Neurosciences* 38(1), pp. 80–88.

You, S., Petrov, T., Chung, P.H. and Gordon, T. 1997. The expression of the low affinity nerve growth factor receptor in long-term denervated Schwann cells. *Glia* 20(2), pp. 87–100.

Zhang, H., Yang, R., Wang, Z., Lin, G., Lue, T.F. and Lin, C.-S. 2011. Adipose tissue-derived stem cells secrete CXCL5 cytokine with neurotrophic effects on cavernous nerve regeneration. *The Journal of Sexual Medicine* 8(2), pp. 437–446.

Zhou, Q., Wang, S., Anderson, D.J. 2000. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron* 25(2), pp. 331–343.

LEGENDS FOR FIGURES AND FIGURE SUPPLEMENTS

Figure 1; Figure Supplement 1:

Restoring Schwann cell c-Jun protein reverses the age-related decline in nerve regeneration.

(A) Representative Western blots of c-Jun in young and aging WT nerves and aging c-Jun OE/+ nerves 3 days post injury. The graph shows densitometric quantitation of the Western blots. c-Jun up-regulation is impaired in WT aged nerves but restored in aged c-Jun OE/+ nerves. Data are normalised to young WT 3 days post cut, * $p < 0.05$, ** $p < 0.005$, ns, non-significant. Young UI WT $n=6$, $n=5$ for all other experimental groups. (B) RTqPCR analysis of 3 day cut nerves. Data normalised to young WT 3 days post cut. ** $p < 0.005$, ns, non-significant, $n=4$. (C) Representative images showing immunofluorescence of c-Jun in double labeling with Schwann cell nuclear marker Sox10 in sections of the distal nerve stump in young and aging WT and aging c-Jun OE/+ mice 3 days post cut. In the graph, the results are quantitated by cell counting. In aging WT Schwann cells, c-Jun is reduced, but elevated

to youthful levels in aging c-Jun OE/+ Schwann cells. *** $p < 0.001$, ns, non-significant. $n = 3$ for each experimental group. (D) Representative images showing Fluorogold-labeled sensory neurons in L4 DRGs of aging WT and c-Jun OE/+ mice 1 week post back-filling following a 4 day crush injury. The graph shows quantitation by cell counting. There is an age-related decrease in back-filled neurons in WT samples ($p = 0.0309$), while the high number of regenerating neurons in young WT mice is maintained in aging c-Jun OE/+ DRGs ($p = 0.0211$). Unpaired Student's t-test. Young WT $n = 6$, Aging WT $n = 5$, Aging c-Jun OE/+ $n = 6$. (E) Representative images of Fluorogold-labeled motor neurons in aging WT and c-Jun OE/+ mice 1 week post back-filling following a 4 day crush injury. The graph shows quantitation of the results. Counts of labeled motor neurons mirror those of sensory neurons since in WT mice, but not in c-Jun OE/+ mice, the number of back-labeled motor neurons decreases with age. *** $p < 0.001$, ** $p < 0.005$, $n = 6$ for all experimental groups. (F) Counts of calcitonin gene-related peptide (CGRP)+ regenerating axons 3mm from crush injury of the sciatic nerve of young and aged WT mice, and of aging c-Jun OE/+ mice. **** $p < 0.0001$, *** $p < 0.001$. Young WT $n = 5$, aging WT and c-Jun OE/+ $n = 6$. All numerical data are analysed by one-way ANOVA with Tukey's multiple comparison test and represented as means \pm SEM. All scale bars: 100 μ m.

Figure 2; Figure Supplement 1:

The mouse model of chronic denervation

(A) Analysis of RNA sequencing data showing decrease in gene expression during chronic denervation. (B) Representative Western blot showing p75NTR expression in uninjured (UI) nerves and distal nerve stumps following 3 days, and 1, 3, 6, 8 and 10 weeks of denervation. The graph shows quantitation of the results. P75NTR peaks 1 week after injury and gradually declines during prolonged denervation. Data normalized to 1 week after injury. One-way ANOVA with Dunnett's multiple comparison test; ** $p < 0.005$, *** $p < 0.001$. $n = 4$. (C) Counts of back-filled Fluorogold-labeled regenerating motor neurons following immediate repair or chronic 10 week denervation show a decrease in motor neuron regeneration into

chronically denervated stumps. Unpaired Student's t-test; ** $p=0.0020$. $n=6$ for each time point. (D) Counts of neurofilament+ axons mirrors the decline in regeneration observed with chronic denervation shown in C. Counts were performed on transverse sections taken 3mm from the repair site 1 week after repair. Unpaired Student's t-test; **** $p<0.0001$. Immediate repair $n=5$, chronic denervation $n=4$. (E) Counts of back-filled Fluorogold-labeled motor neurons showing similar numbers of regenerating neurons following immediate repair or repair after 1 week of denervation. Unpaired Student's t-test; $p=0.9$. $n=3$. All numerical data represented as means \pm SEM.

Figure 3:

c-Jun declines in the distal nerve stump during chronic denervation and long-term culture

(A) Representative Western blot of c-Jun in WT uninjured (UI) nerves and distal stumps following 3 days and 1, 3, 6, 8 and 10 weeks of denervation. The graph quantitates the results, showing an initial increase followed by a decline in c-Jun levels. Data normalized to 1 week post injury. One-way ANOVA with Dunnett's multiple comparisons test; * $p<0.05$, ** $p<0.005$, **** $p<0.0001$. $n=5$. (B) Representative Western blot comparing c-Jun expression 1 week after injury in WT and c-Jun cKO mice. The graph quantitates the results, showing up-regulation of c-Jun in WT nerves but not in c-Jun cKO nerves, demonstrating that the c-Jun up-regulation after injury is Schwann cell specific. Data normalized to WT 1 week post injury. Two-way ANOVA with Sidak's multiple comparison test; **** $p<0.0001$. $n=5$. (C) Representative immunofluorescence images of c-Jun/Sox10 double labeling in transverse sections of the distal stumps 1 and 10 weeks after cut. Boxed areas shown at higher magnification in right hand panels. Note loss of c-Jun protein from Schwann cell nuclei at 10 weeks (arrows). (D) Representative Western blot of c-Jun and p75NTR in Schwann cell cultures after 2 or 9 passages. The results are quantitated in the graphs, showing decline in c-Jun and p75NTR with time *in vitro*. Data normalised to passage 2. Unpaired Student's t-test; ** $p=0.0023$, *** $p=0.0007$. $n=6$ for c-Jun, $n=7$ for p75NTR (E) qPCR analysis showing

reduction in c-Jun mRNA in Schwann cultures following 9 passages. Data normalized to passage 2. Unpaired Student's t-test; * $p=0.0299$. $n=3$. (F) c-Jun/Sox10 double labeling with nuclear marker DAPI after 2 or 9 passages. Note decline of nuclear c-Jun in passage 9 cells. All numerical data represent means \pm SEM.

Figure 4:

c-Jun expression is maintained in c-Jun OE/+ Schwann cells during chronic denervation

(A) Representative Western blots of c-Jun in WT and c-Jun OE/+ distal stumps after 1, 3 and 10 weeks of denervation. The results are quantitated in the graph. In contrast to WT nerves, c-Jun OE/+ nerves maintain consistent levels of c-Jun during 10 week chronic denervation. Data normalized to WT 1 week post injury. Two-way ANOVA with Sidak's multiple comparisons test; * $p<0.05$. $n=5$. (B) Representative images showing c-Jun/Sox10 double immunofluorescence in transverse sections of WT and OE/+ uninjured and injured distal stumps. The graph shows quantitation by cell counting. The c-Jun labeling of Sox10 positive nuclei in the two genotypes is comparable at 1 week, but reduced at 10 weeks in WT nerves only. Two-way ANOVA with Tukey's multiple comparison test; *** $P<0.001$. $n=3$. All numerical data represented as means \pm SEM, all scale bars: 100 μ m.

Figure 5:

The regenerative capacity of c-Jun OE/+ nerves is maintained during chronic denervation

(A) Representative images showing Fluorogold-labeling of neurons in L4 DRGs of WT and c-Jun OE/+ mice after 2 weeks of regeneration into acutely transected (immediate repair) or chronically denervated (10 weeks) distal stumps. Quantification by cell counting is in the graph. The number of back-filled DRG neurons following regeneration through chronically denervated WT stumps was reduced, but maintained after regeneration through chronically denervated c-Jun OE/+ stumps. One-way ANOVA with Tukey's multiple comparison test; ** $p<0.005$, * $p<0.05$, ns non-significant. WT immediate repair and chronic denervation $n=6$, OE/+ chronic denervation $n=8$. (B) Representative images showing Fluorogold- labeling of

back-filled motor neurons in WT and c-Jun OE/+ mice after 2 weeks of regeneration into acutely transected (immediate repair) or chronically denervated distal stumps. Quantification is in the graph, showing that compared to immediate repair, the number of labeled neurons is reduced after regeneration through chronically denervated WT stumps, but not after regeneration through chronically denervated c-Jun OE/+ stumps. One-way ANOVA with Tukey's multiple comparison test; $**p<0.005$. WT immediate repair and chronic denervation $n=6$, c-Jun OE/+ chronic denervation $n=8$. (C) Counts of neurofilament+ immunofluorescent fibers in the distal stump 1 week after repair following chronic denervation of WT and c-Jun OE/+ nerves. In parallel experiments, the number of neurofilament+ fibers in WT nerves after immediate repair was about 200 (see Fig.2 D). Unpaired Student's t-test; $*p=0.0334$. WT $n=4$, c-Jun, OE/+ $n=3$. (D, E) Counts of Fluorogold-labeled sensory (D) and motor (E) neurons in WT and c-Jun OE/+ mice following transection with immediate application of tracer. The number of back-filled sensory ($p=0.1872$) and motor ($p=0.7153$) neurons is similar. Unpaired Student's t-tests, $n=3$ for each experimental condition. (F) The number of back-filled sensory neurons in WT and c-Jun OE/+ mice is similar after transection followed by immediate repair. Unpaired Student's t-test; $p=0.9195$. $n=3$. (G) The number of labeled motor neurons in WT and c-Jun OE/+ mice is similar when tracer was applied 5 days after sciatic nerve crush. Unpaired Student's t-test; $p=0.312$. WT $n=5$, OE/+ $n=4$. All numerical data represented as means \pm SEM, all scale bars: 100 μ m.

Figure 6; Source Data 1

Cell number and nerve size in injured WT and c-Jun OE/+ nerves

Cell nuclei were counted in whole transverse profiles of the tibial nerve, 5mm from the injury site, using the electron microscope. (A) Schwann cell numbers in young and aged WT and c-Jun OE/+ nerves. (B) Macrophage density and (C) number in young and aged WT and c-Jun OE/+ nerves. (D) Fibroblast density and (E) number in young and aged WT and c-Jun OE/+ nerves: $*p<0.05$. (F) Whole transverse profiles of the tibial nerve were measured for the nerve area of young and aged WT and c-Jun OE/+ nerves: $**p<0.005$. For counts in A-F,

n=4 for each condition. (G) Schwann cell numbers in 2 and 10 week cut nerves of WT and c-Jun OE/+ mice; *p<0.05, **p<0.005, ****p<0.0001. (H) Macrophage density and (I) number in 2 and 10 week cut nerves of WT and c-Jun OE/+ mice: **p<0.005, ***p<0.001. (J) Fibroblast density and (K) number in 2 and 10 week cut nerves of WT and c-Jun OE/+ mice: *p<0.05, **p<0.005. (L) Whole transverse profiles of the tibial nerve were measured for the nerve area of 2 and 10 week cut nerves of WT and c-Jun OE/+ mice: *p<0.05, ****p<0.0001. For counts in G-L, 2 week WT and c-Jun OE/+ n=7, 10 week WT and c-Jun OE/+ n=5. All number data represented as means \pm SEM analysed by two-way ANOVA with Tukey's multiple comparison test.

Figure 7:

Sonic hedgehog promotes c-Jun activation in Schwann cells in vivo and in vitro

(A) Representative Western blot showing c-Jun expression in WT and Shh cKO (cKO) nerves 3 and 7 days after cut. Quantitation is shown in the graph where the data are normalized to WT 3 days post cut. Two-way ANOVA with Sidak's test; *p<0.05. n=5 for each genotype. (B) Representative Western blot showing c-Jun and phosphorylated c-Jun (p-c-Jun) in WT and Shh cKO distal nerve stumps 7 days post cut. Quantitation is shown in the graph where the data are normalized to WT 7 days post cut. Unpaired Student's t-test; **p=0.0014, n=5 for each genotype. (C) Representative Western blot showing p75NTR protein in WT and Shh cKO nerves 7 days post cut. The graph shows quantitation of the results. Data are normalized to WT 7 days post injury. Unpaired Student's t-test; ****p=<0.0001. n=5 for each genotype. (D) Representative Western blot showing c-Jun in Schwann cell cultures exposed to SAG for 48 hours. (Veh: DMSO vehicle). Quantitation is shown in the graph where the data are normalized to vehicle. One-way ANOVA with Dunnet's test; *p<0.05, **p<0.005. n=6. (E) Representative Western blot showing c-Jun in Schwann cell cultures exposed to purmorphamine for 48 hours. Quantitation is shown in the graph where the data are normalized to vehicle. One-way ANOVA with Dunnet's test;

*p<0.05. n=3. (F) Representative immunofluorescence images showing increased c-Jun labeling of Sox10 positive Schwann cell nuclei after 48 hours incubation with SAG compared to vehicle (DMSO). Scale bar: 100µm.

Figure 8:

Sonic hedgehog plays a role in c-Jun activation in Schwann cells via autocrine signaling

(A, B) qPCR showing mRNA expression of (A) *Bdnf* *p=0.0314 and (B) *Gdnf* *p=0.0382 in Schwann cell cultures incubated for 48 hours with SAG. Data normalized to vehicle. Unpaired Student's t-tests. n=4 for each condition. (C) Differential interference contrast (DIC) microscopy showing changes in Schwann cell morphology after 48 hours incubation with purmorphamine (DMSO vehicle). Scale bar: 50µm. Graphs depict changes in cell area, roundness and length/√area following incubation with purmorphamine, demonstrating enhancement of elongated morphology. One-way ANOVAs with Tukey's multiple comparison test ***p<0.001. n= 3, each experiment involving measurement of 100 cells per condition. (D) Representative Western blots showing c-Jun and phosphorylated c-Jun in cultured Schwann cells after 48 hours incubation with cyclopamine alone (DMSO vehicle). The graphs show quantitation of the blots. Data are normalized to vehicle. One-way ANOVA with Dunnet's multiple comparisons test; **p<0.005; ***p<0.001. n=3. All numerical data represented as means ± SEM.

Figure 9; Figure Supplement 1:

Bioinformatics analysis of RNA seq. data from young and aged nerves

(A) Over-representation analysis showing enrichment of c-Jun regulated genes in various WT injury paradigms. p= 3.2x10⁻⁸ for UI young vs aged; p=1x10x⁻⁷ for 3 day cut young vs aged; p=2.3x10⁻¹³ for the injury response. p-values computed by one-sided Fisher's exact test. (B) GO terms down-regulated and (C) up-regulated in uninjured nerves of aged WT mice. (Absolute fold change >2 and FDR<0.05). (D) GO terms down-regulated and (E) up-regulated in the injury response of aged WT mice. (Absolute fold change >2 and FDR<0.05).

(F) When aged c-Jun OE/+ and WT nerves are compared, genes associated with protein processing (FDR = 0.00318) and maturation (FDR = 0.0153) are significantly enriched in aged c-Jun OE/+ nerves compared to aged WT. (G) Venn diagram showing numbers of differentially expressed genes between young and aged 3 day cut WT nerves and aged 3 day cut OE/+ nerves, compared to their uninjured counterparts. (H) Mean expression of 4 c-Jun regulated genes with significantly different expression between young and aged WT nerves but not between young WT and aged c-Jun OE/+ nerves. (Absolute fold change >2 and FDR<0.05).

Figure 9 - Figure Supplement 1

Bioinformatics analysis of aged and young nerves following injury. (A) Principal component analysis (PCA) shows that the key source of variance in our aging analysis is injury status with samples clustering together after injury (red) regardless of age or genotype. (B) Gene set enrichment analysis (GSEA) of uninjured young and aged WT nerves showed enrichment in the down-regulation of c-Jun regulated genes in aged nerves. Normalized enrichment score (NES) = -1.85, q-value = 0.0. (C) GSEA analysis comparing injured old and young nerves showed a similar enrichment of c-Jun genes in the down-regulation of these genes, suggesting a failure of c-Jun regulation in aged nerves. NES= -1.61, q-value = 0.0. (D) GSEA of young and aged nerves compared to their uninjured controls and then to each other to examine the differential expression between the two in response to injury. Analysis shows that c-Jun gene up-regulation was highly enriched by injury. NES= 2.01, q-value = 0.0.

Figure 10; Figure Supplement 1:

Bioinformatics analysis of RNA seq. data from acutely and chronically denervated nerves

(A) GO terms down-regulated and (B) up-regulated in WT nerves during chronic denervation. (C) When chronically denervated c-Jun OE/+ and WT nerves were compared, GO terms associated with Schwann cell differentiation (FDR = 0.00397) and PNS

development (FDR = 0.0173) were enriched in c-Jun OE/+ nerves. (D) Venn diagram showing numbers of differentially expressed genes between WT nerves following acute (1 week) and chronic (10 week) denervation and c-Jun OE/+ nerves following chronic denervation, compared to their uninjured counterparts. (E) Mean expression of 5 c-Jun regulated genes with significantly different expression between acute and chronic WT nerves, but not between acute WT and chronic c-Jun OE/+ nerves. (Absolute fold change >2 and FDR<0.05).

Figure 10 - Figure Supplement 1

(A) PCA showing the key sources of variance in our chronic injury analysis are not only injury dependent but also time post injury dependent, with samples clustering based on length of denervation regardless of genotype. (B) GSEA of WT chronically denervated nerves showed that c-Jun genes were some of the most down-regulated genes during chronic denervation NES = -2.5, q-value = 0.0.

SUPPORTING FILES

Supplementary File 1: The 15 most regulated genes in the tibial nerve of WT mice during aging. The data compare (A) uncut nerves, (B) 3 day cut nerves and (C) the injury response. In (A) and (B), genes expressed at higher levels in aged mice under the conditions indicated are in blue (top), while genes with reduced expression in aged mice are in red (bottom). In(C), genes that respond more strongly to injury in aged mice are in blue (top), while genes with weaker injury response in aged mice are in red (bottom).

Supplementary File 2: All significantly regulated genes in the tibial nerve of WT mice during aging and chronic denervation. (A, B) Genes expressed at higher levels in aged mice under the conditions indicated are in blue (top), while genes with reduced expression in

aged mice are in red (bottom). (C) Genes that respond more strongly to injury (3 day cut vs UI) in aged mice are in blue (top), while genes with weaker injury response are in red (bottom). (D) The 303 genes that are significantly expressed in young WT vs UI and aged c-Jun OE/+ vs UI 3 days after injury. (E) Genes expressed at higher levels after chronic denervation are in blue (top), while genes with reduced expression are in red (bottom). (F) The 227 genes that are significantly expressed in WT 1 week vs UI and c-Jun OE/+ 10 week vs UI.

Supplementary File 3: 138 genes regulated by c-Jun in injured nerves derived from Arthur-Farraj et al. (2012). Blue (top) indicates genes expressed at higher levels in cut nerves of WT mice compared with nerves of mice with conditional c-Jun inactivation selectively in Schwann cells (92 genes), while red (bottom) indicates genes expressed at lower levels in cut WT nerves compared to c-Jun mutant nerves (46 genes).

Supplementary File 4: The 15 most regulated genes in the tibial nerve during chronic denervation. Genes expressed at higher levels after chronic denervation are in blue (top), while genes with reduced expression are in red (bottom).

1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292